RECOGNITION OF HEPATITIS B EPITOPES BY T CELL RECEPTORS AND T CELL RECEPTOR-LIKE ANTIBODIES

BY

JIANRONG LIONEL LOW

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biochemistry
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

Doctoral Committee:

Professor David M. Kranz, Chair
Professor David J. Shapiro
Associate Professor Raven H. Huang
Associate Professor Chad M. Rienstra

ABSTRACT

T cells play a vital role in the adaptive immune response against viral infections. Virus-specific T cells recognize infected cells through a heterodimeric surface receptor known as the αβ T cell receptor (TCR). The TCR recognizes a peptide epitope presented by a product of the major histocompatibility complex (MHC). All nucleated cells express class I MHC products, typically in association with one of thousands of "self" peptides. However, during a viral infection, "foreign" peptides derived from the expression of viral proteins would also be presented on the cell surface. T cells expressing TCRs that specifically recognize these viral peptide-MHC complexes (pMHC) can become activated and destroy the virus-infected cell. However, the immune system of many patients with chronic viruses has undergone a process of tolerance whereby the T cells are either non-existent or inactive. The ability to study the viral pMHC on infected cells would allow a greater understanding of anti-viral responses and the development of specific targeting molecules against these pMHC could lead to the generation of novel therapeutic interventions to control viral infections. The studies described here focus on these goals.

Chapter 2 describes the characterization of two monoclonal antibodies that are specific for immuno-dominant hepatitis B virus epitopes, the core protein peptide 18-27 ($Core_{18-27}$) and the envelope protein peptide 183-191 ($Env_{183-191}$), both restricted by the human HLA-A*02:01 class I MHC. Both murine monoclonal antibodies exhibited specificity and high affinity for their

respective epitopes. In addition, the antibodies were able to recognize pMHC generated exogenously by pulsing HLA-A*02:01^{+} T2 cells with the respective peptides, and also endogenous pMHC complexes produced by HBV infected cell lines. The antibodies were used further to quantify and visualize specific HBV pMHCs on the surface of various cell lines, allowing the study of how density and distribution of these pMHC ligands affected T cell activation.

In Chapter 3, I sought to examine the use of soluble TCRs in comparison with a monoclonal antibody generated against the same antigen. To approach the question, the α and β chain genes of a TCR specific for the envelope protein peptide 183-191 ($Env_{183\text{-}191}$) epitope were cloned from a T cell line and the extracellular domains were expressed in *Escherichia coli.* Characterization of the refolded, soluble TCR showed that only tetrameric forms bound specifically to $Env_{183\text{-}191}$ pMHC in a surface plasmon resonance assay, or with peptide-pulsed target cells, consistent with the well-known low affinities of TCRs. To further explore the specificity of the TCR, I developed a sensitive bead-based avidity assay. Using this assay, a comparison between the TCR tetramer and the $Env_{183\text{-}191}$ specific monoclonal antibody showed that although the tetrameric form of the soluble TCR had similar functional avidity for the $Env_{183\text{-}191}$ pMHC as the monoclonal antibody, it required a higher density of pMHC ligands in order to achieve significant binding. Nevertheless, the assay revealed a difference in fine specificity of the TCR and the monoclonal antibody, consistent with evidence that the T cell clone could recognize a particular virus-variant (clade), which the antibody could not recognize.

Finally in Chapter 4, I used the sensitivity of the avidity-based assay described in Chapter 3 to develop a novel method for the *in vitro* engineering of T cell receptors expressed in a yeast display system. In view of the limitations imposed by the low affinity of soluble TCRs, protein engineering is necessary to improve the monomeric affinity (a process called affinity maturation). Conventional methods for screening yeast-displayed libraries of TCRs had used tetrameric pMHC or commercially available dimeric MHC-Immunoglobulin fusion molecules. To improve the sensitivity of the screening strategy, the bead-based assay was applied to a yeast display system with model TCRs of varying affinities. The pMHC-coated beads were able to significantly stain lower affinity TCRs that had previously not been isolated using the tetrameric and dimeric reagents. Furthermore, the assay was used in the screening of a single-chain TCR library, isolating not only high affinity mutants but also intermediate affinity mutants that would not have been selected by the other reagents. In the absence of high affinity solutions within libraries, these intermediate affinity mutants can be potential leads with which further engineering can be done, thus increasing the success rate of TCR engineering. They could also serve as TCRs with improved activities in adoptive T cell therapies, which are known to benefit from such intermediate affinity TCRs.

The molecular probes available to specifically target peptide-major histocompatibility complexes are relatively uncommon and thus the capacity to study pMHC distribution and quantities has been limited. The key problems have been due to the fact that the natural receptors for the pMHC, the T cell

receptors, are difficult to express in soluble form and are of poor affinity. TCR-like antibodies against a specific pMHC provide one possible solution, but they are difficult to generate, and their specificities are often not adequate (e.g. they bind MHC epitopes such that the peptide contribution to binding varies). In this thesis, I describe the characterization and applications of two such antibodies and a soluble TCR that are able to specifically target HBV pMHCs. I also describe the development of a new screening assay that would facilitate yeast display protein engineering in the generation of high affinity TCRs specific for pMHC. These probes provide opportunities to study the details of viral peptide presentation and anti-viral immune responses, and they also provide potential molecular therapies, with targeted delivery capacities that would aid the treatment and resolution of viral infections.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors, Antonio Bertoletti from Singapore Institute for Clinical Sciences and David Kranz, for accepting me into their mentorship. My interest in immunology developed during my undergraduate years in Imperial College London and while I was looking forward to pursuing further studies in immunology for my graduate education, I did not anticipate that I would have the opportunity to learn from two outstanding scientists and leaders in their fields. Both Antonio and Dave had provided me with their unwavering support and guidance, the opportunity to broaden my knowledge of immunology from two unique perspectives, and more importantly, the excellent training and development into a scientist. They had taught me the importance of being critical and analytical in thinking and to approach every problem and challenge with an open mind. Their doors were always open for discussions or advice; while their enthusiasm and insights had spurred me to think outside the box while not losing focus.

I would also like to thank Dr Gijsbert Grotenbreg from the National University of Singapore. Gijs had been generous in allowing me to carry on with my work on the T cell receptors, that I had started in UIUC, in his laboratory in Singapore. Gijs had been more than a collaborator during my time in his laboratory. He had been a great tutor; teaching me the importance of tackling problems in a systematic and analytic manner, and also an inspiring scientist whose enthusiasm was infectious and uplifting.

I owe the success of my graduate work to the many wonderful people whom I have had the opportunity to work with during these years. Firstly, I would like to acknowledge the support and help I received in the Antonio Bertoletti laboratory. I especially like to thank Konduro Sastry with whom I worked closely with during the TCR-like antibody project. I would also like to thank Adam Gehring, Denise Teoh, Juandy Jo and Antony Chen for the numerous insightful discussions as well as their constant support and advice. In addition, I would like to thank Adeline Chia and Zack Ho for their patience while training me in cell culture work. They had never hesitated to share their knowledge or provide advice whenever I faced problems or had questions.

It had been a truly fun and memorable experience working with the wonderful members of Gijs' group. I would like to thank Joanna Choo, Michelle Foo, Kenneth Wong, Kai Yee Toh and Ming Yan Or for their help and advice. Their readiness to offer any help or advice and the numerous lively and insightful discussions made me feel like a member of the laboratory. I would especially like to thank Gladys Yeo and Anneta Naidoo for their invaluable contributions and help with the work on the soluble T cell receptors, and also Zhenwei Tan for the sharing and troubleshooting sessions where we shared both our problems and solutions while working on the receptors.

The fantastic members of the Kranz laboratory played a huge part in my journey through graduate school. Jennifer Stone had been a wonderful mentor, never failing to provide invaluable advice and suggestions whenever I hit a stumbling block. I would also like to thank Dave Aggen and Beth Marshall for

sharing their valuable experiences and advice during my work with yeast display. I would also like to acknowledge Adam Chervin, Sheena Smith, Ningyan Wang, Daiva Mattis, Sami Narayanan and the members of Ed Roy's laboratory for all their help and support during these years.

Last but not least, I would like to thank my family who has been understanding and supportive during these long years. They provided me with listening ears and were never short on encouraging words during my years in graduate school.

TABLE OF CONTENTS

LIST OF TABLES xii

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xv

CHAPTER ONE

INTRODUCTION 1
The immune system 1
B cells and antibodies 4
T cells and T cell receptors 6
Antigen processing and presentation 10
The Hepatitis B virus 14
HBV biology and antigens 15
Immune responses to HBV infection 17
Chronic HBV infections and hepatocellular carcinoma 19
Engineering TCRs and antibodies against HBV pMHCs 21
Soluble T cell receptors 22
TCR-like antibodies 23
In vitro engineering of T cell receptors 24
Yeast display engineering 26
Figures 27

CHAPTER TWO

CHARACTERIZATION OF T CELL RECEPTOR-LIKE ANTIBODIES SPECIFIC FOR HEPATITIS B ANTIGENS 29
Introduction 29
Materials and methods 32
Generation of TCR-like antibodies 32
Cell lines 33
Detection of pMHC complexes on pulsed and HBV producing cells 33
Surface plasmon resonance 34
Quantification of pMHC complexes 34
HBV-specific $CD8^+$ T cells degranulation assay 35
Visualization of pMHC complexes by fluorescence and confocal microscopy 35
Results 36
Specificity of TCR-like antibodies 36

Binding avidity of TCR-like antibodies 37
Fine specificities of TCR-like antibodies 38
TCR-like antibodies recognize endogenously processed and presented pMHC in HBV infected cells....39
Visualization of pMHC complexes in pulsed cells and virus producing cell lines by confocal microscopy....40
Quantifying pMHC complexes required for T cell activation on pulsed cells....42
Effect of interferons α and γ on antigen presentation....44
Discussion 45
Figures....50

CHAPTER THREE

COMPARISON OF A SOLUBLE T CELL RECEPTOR AND A T CELL RECEPTOR-LIKE ANTIBODY SPECIFIC FOR HEPATITIS B/HLA-A2 59
Introduction....59
Materials and methods 61
Cloning of TCR α and β chains....61
Expression of TCR chains 62
Refolding and purification of refolded TCRs 62
Multimerization of refolded TCRs 63
TCR-like antibody 63
Peptide-MHC conjugated beads assay 64
Cell culture and staining 65
Surface plasmon resonance....65
Structural modelling....66
Results....66
*Producing soluble $Env_{183-191}$ peptide/HLA-A*02:01-specific TCR tetramers*....66
A bead-based assay to probe pMHC binding to soluble TCR tetramers....67
TCR tetramers and the analogous TCR-like mAb have distinct fine-specificities 68
Detection limit of the bead-based assay....70
*TCR tetramers bind cells only with high density of $Env_{183-191}$/HLA-A*02:01 complexes*....71
Discussion 72
Figures....76

CHAPTER FOUR

DEVELOPMENT OF PEPTIDE-MHC COATED BEADS FOR DETECTION OF T CELL RECEPTORS IN A YEAST DISPLAY SYSTEM....86
Introduction....86

Materials and methods 90
Yeast display of single-chain and full-length T cell receptors....90
In vitro refold and biotinylation of pMHC complexes....91
Production of and yeast cell staining with pMHC beads 92
Fluorescence-activated cell sorting of de novo library....92
Results....93
Production of pMHC-coated beads....93
pMHC beads have higher sensitivity than dimeric or tetrameric pMHCs....94
Sensitivity of pMHC staining varies with beads concentration and pMHC density *96*
Selection of TCRs with intermediate and high affinities from a scTCR library with pMHC beads....98
Isolation and characterization of Tax/A2 binding clones....98
Screening for HBV C18/A2 specific TCRs from a full-length TCR library with pMHC beads....100
Discussion 103
Figures....108

REFERENCES....117

LIST OF TABLES

Table 3.1 Quantification of pMHCs presented on streptavidin-coated beads.....85

Table 4.1 HBV Core $_{18\text{-}27}$ specific TCR CDR3α library design 116

LIST OF FIGURES

Figure 1.1 Immune receptors of the B cell and the T cell 27

Figure 1.2 Structure of hepatitis B virion and genome 28

Figure 2.1 Generation of monoclonal antibody with TCR-like specificity for HBV $Core_{18-27}$ and $Env_{183-191}$ HLA-A2 peptide MHC complex. 50

Figure 2.2 Specificity of TCR-like antibodies that recognize 2 hepatitis B virus peptides restricted by the HLA-A2 MHC molecule 51

Figure 2.3 Measuring the dissociation constant (K_D) of the TCR-like antibodies 52

Figure 2.4 Comparison of fine specificity of TCR-like antibodies and respective T cell receptors 53

Figure 2.5 TCR-like antibodies recognize and bind both exogenously pulsed and endogenously process pMHC complexes 54

Figure 2.6 Visualization of endogenously processed and exogenously pulsed $Core_{18-27}$ pMHC on the surface of cells.....55

Figure 2.7 Visualization of $Env_{183-191}$ pMHC on the surface of infected cells and internalization of Env183/A2 antibody.....56

Figure 2.8 Quantifying pMHC complexes required for T cell activation on pulsed cells.....57

Figure 2.9 Effect of interferons alpha and gamma on the presentation of specific pMHCs 58

Figure 3.1 FPLC purification profiles of *in vitro* refolded soluble TCR.....76

Figure 3.2 Protein analysis of purified soluble full length TCR.....77

Figure 3.3 Peptide MHC complex coated beads as an artificial antigen-presenting cell. 78

Figure 3.4 Soluble TCR binds specifically to $Env_{183-191}$ pMHC and recognizes both natural variants Genotypes A/C/D and Genotype B. 79

Figure 3.5 Comparison of fine specificity of Env183/A2 antibody and soluble TCR tetramers 80

Figure 3.6 Functional avidity of the Env183/A2 antibody and soluble TCR tetramers 81

Figure 3.7 Peptide-MHC sensitivity comparison of Env183/A2 antibody and soluble TCR tetramer 82

Figure 3.8 Recognition of exogenously pulsed pMHC presented on the surface of cells 83

Figure 3.9 Recognition of endogenously presented pMHC complexes...84

Figure 4.1 Production of pMHC beads 108

Figure 4.2 Detection of yeast displayed single chain T cell receptors with multimeric pMHC detection reagents 109

Figure 4.3 Staining of yeast displayed 2C TCR and higher affinity mutants with pMHC beads presenting SIY peptide variants 110

Figure 4.5 Sensitivity of pMHC beads 111

Figure 4.5 Sorting an A6 scTCR library with Tax/A2 beads 112

Figure 4.6 Analysis of clones isolated from A6 scTCR library using Tax/A2 pMHC beads 113

Figure 4.7 Beads and tetramer titration analysis of 3 strong and 3 intermediate binding clones.....114

Figure 4.8 Sorting a full length C18/A2 TCR library sorting with C18/A2 beads.....115

LIST OF ABBREVIATIONS

ABC – Adenosine triphosphate-binding cassette
ADCC – Antibody-dependent cell-mediated cytotoxicity
AHR – Airway hyper-responsiveness
AIRE – Autoimmune regulator
APC – Antigen presenting cell
β2m – Beta-2 microglobulin
BCR – B cell receptor
BSA – Bovine serum albumin
cccRNA – Covalently closed circular DNA
Core18 – HBV core 18-27 peptide
Cα – α chain constant domain of TCR
Cβ – β chain constant domain of TCR
CDR – Complementarity determining region
CLIP - Class II-associated invariant chain peptide
CRP – C-reactive protein
CTL – Cytotoxic T lymphocytes
CXCL – Chemokine (C-X-C motif) ligand
DAPI – 4',6-diamidino-2-phenylindole
DED – Death effector domain
DISC – Death-inducing signaling complex
DTH – Delayed-type hypersensitivity
DTT – Dithiothreitol
EDC – N-ethyl-N'-[3-(diethylamino)propyl]carbodiimide
EDTA – Ethylenediaminetetraacetic acid
ER – Endoplasmic reticulum
Env183 – HBV surface (envelope) 183-191 peptide
Erp57 - Thiol-disulfide oxidoreductase found in ER
Fab – Fragment antigen binding
FBS – Fetal bovine serum
FADD – Fas-associated protein with Death Domain
Fc – Fragment crystalizable
GATA-3 – GATA binding protein 3, Th_2 specific transcription factor
HBV – Hepatitis B virus
HBsAg – Hepatitis B surface antigen
HBcAg – Hepatitis B core antigen
HBeAg – Extracellular form of HBcAg
HBx – Hepatitis B X protein
HCC – Hepatocellular carcinoma
HLA – Human leukocyte antigen
HTLV – Human T-cell lymphotrophic virus
IFN - Interferon
Ig - Immunoglobulin
IL – Interleukin
iT_{reg} – Induced regulatory T cell

LPS - Lipopolysaccharide
mAb – Monoclonal antibody
MASP – MBL-associated serine proteases
MBL – Mannan-binding lectin
MEM – Minimal essential medium
MHC – Major histocompatibility complex
NHS – N-hydroxysuccinimide
NK – Natural Killer cells
nT_{reg} – Natural regulatory T cell
ORF – Open reading frame
PAMPs – Pathogen associated molecular patterns
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PFA - Paraformaldehyde
pMHC – peptide-MHC complex
PRR – Pattern recognition receptors
RAG – Recombination activating gene
rcDNA – Relaxed circular DNA
RORγt – Retinoic acid receptor-related orphan receptor gamma expressed by thymocytes, Th_{17} specific transcription factor
RT – Room temperature
scTCR – Single chain TCR
SPR – Surface plasmon resonance
TAP – Transporters associated with antigen processing
T-bet – T-box expressed in T cells, Th_1 specific transcription factor
TCR – T cell receptor
TGF – Transforming growth factor
T_{fh} – Folicular helper T cell
Th_1 – T helper cell type 1
Th_2 – T helper cell type 2
Th_9 – IL-9 secreting T helper cell
Th_{17} – IL-17 secreting T helper cell
Th_{22} – IL-22 secreting T helper cell
T_{reg} – Regulatory T cell
TLR – Toll-like receptors
TNF – Tumor necrosis factor
Vα – α chain variable domain of TCR
Vβ – β chain variable domain of TCR
V_H – Heavy chain variable domain of antibody
V_L – Light chain variable domain of antibody

CHAPTER ONE

INTRODUCTION

The Immune System

The human body is constantly exposed to parasites, bacteria and viruses. However, infection is largely prevented with the help of the highly sophisticated immune system. The two branches of the immune system, innate and the adaptive are composed of a variety of cells and a myriad of proteins working together to recognize and eliminate these infectious pathogens. While the innate immune system represents the first line of defense against pathogens, the adaptive immune system involves a pathogen specific response to eradicate the infection and also provide long-term immunity through immunological memory. Although both systems differ in how they confer immunity against invading pathogens, immune responses elicited by either system have significant influence over the other and they work together to prevent pathogenic infections.

The innate immune system is the evolutionarily more ancient branch of the immune system; for example, both insects and mammals share similarities in pathogen recognition, signalling pathways, and effector mechanisms (93). Innate responses to infection are the first to develop, are generally not antigen specific, and react broadly to various pathogens. Components of the innate immune system range from physical barriers to pathogens, such as skin epithelium and mucus layers in the respiratory and gastrointestinal tracts, to soluble mediators such as defensins and complement

proteins, to immune cells such as macrophages, neutrophils, eosinophils and natural killer (NK) cells that elicit a variety of distinct immune responses (60, 74, 101).

The major requirement in an immune response is the ability of immune cells to differentiate between host (self) and foreign (non-self) components. Innate immune cells recognize pathogens through the expression of “pattern recognition receptors” (PRRs) such as mannan-binding lectin (MBL), C-reactive protein (CRP) and the Toll-like receptors (TLRs) (72, 125, 194). MBL and CRP are associated with the activation of the complement pathway in the innate system. MBL binds to terminal mannose residues found on the surface of many microorganisms, and associates with the MBL-associated serine proteases (MASP) MASP1 and MASP2. The serine proteases are activated and subsequently they initiate the lectin pathway of complement by cleaving C2 and C4 proteins (60). CRP on the other hand functions as an opsonin, binding to phosphorylcholine on the surface of bacteria, and then with complement C1q, triggering the classical complement pathway (4).

TLRs are a family of receptors with broad specificity, recognizing pathogen associated molecular patterns (PAMPs) that are molecular motifs uniquely associated with pathogens. PAMPs include molecules such as the flagella of bacteria, lipopolysaccharide (LPS) on gram-negative bacteria, teichoic acids on gram-positive bacteria, the un-methylated dinucleotide CpG motif in bacteria DNA, double and single stranded viral RNAs, glycolipids and zymosans of yeast and fungi (194). Immune cells such as macrophages, dendritic cells, and mast cells utilize TLRs to recognize invading pathogens and upon binding, the receptors initiate an inflammatory response that involves the secretion of cytokines such as IL-1, IL6, IL-8, TNF-alpha, type I interferons,

and chemokines that help recruit other immune cells to the site of infection (82, 140, 191). Phagocytic cells such as macrophages and neutrophils also express other receptors such as the complement receptors, scavenging receptors and the Fc receptors (specific for the Fc, “Fragment crystalizable”, region of antibodies) that are able to bind to opsonized pathogens and activate phagocytosis. These cells engulf and internalize pathogens in intracellular phagosomes and subsequently degrade them with an arsenal of molecules such as defensins, lyzosyme, hydrolases and toxic reactive oxygen species including superoxide free radicals, hypochorite, hydrogen peroxide and nitric oxide (27, 84). The adaptive immune system is the more recently evolved branch of the immune system, found only in vertebrates (52). Whereas innate immune responses are not considered specific for each pathogen, adaptive immune responses are highly specific and require considerably more time to develop, especially when a pathogen is encountered for the first time. More importantly, adaptive responses also provide long lasting immune protection after initial contact with pathogens. Adaptive immunity involves two general classes of responses; humoral and cellular immune responses, with B and T lymphocytes being the main effector cell types, respectively (152). As indicated, the essential feature of the immune system is the ability to distinguish between self and non-self. B cells and T cells express B cell receptors (BCRs) and T cell receptors (TCRs), members of the immunoglobulin superfamily, in order to identify foreign pathogens (Figure 1.1) (222). Each B cell and T cell expresses a potentially unique BCR or TCR that is specific for a particular antigen, giving rise to a diverse population of lymphocytes, each having their own specificity. This vast diversity in antigen specific receptors is a product of a unique somatic recombination process

mediated by two recombination activating gene (RAG) enzymes, RAG-1 and RAG-2 (144, 173). It has been hypothesized that during evolution, insertion of transposable elements into primordial immunoglobulin genes generated the gene segments that encode the BCR and TCR (3, 197). Further duplications of the individual gene segments, known as variable (V), diversity (D), joining (J) and constant (C) gene segments, could then have given rise to the genetic diversity of the antigen receptors of the present day B cell receptor and T cell receptor.

B cells and antibodies

B cells and T cells originate from the same hematopoietic stem cells. While T cells develop in the thymus (hence the name), B cells develop in the bone marrow. B cells produce antibodies, molecules that bind to antigens such as pathogens and toxins, thus neutralizing them by mediating antibody-dependent cell-mediated cytotoxicity (ADCC), phagocytosis, and/or activation of complement-mediated immunes responses (156). An antibody has often been illustrated as a “Y” shaped molecule, made up of two heavy chains and two light chains, respectively encoded by the IgH gene containing V, D, J, and C segments and the IgL gene containing V, J, and C segments. Each arm (Fab, for “Fragment antigen binding”) of the antibody contains one variable and one constant domain from each chain forming the antigen binding region, while the tail region (Fc) is made up of constant domains of the heavy chain, which can interact with Fc receptors or complement.

B cells undergo several stages in development during which the genetic recombination of the V, D and J segments at the IgH locus occurs, followed by the V

and J recombination of IgL locus. Three complementarity determining regions (CDRs) are formed by loops of approximately 5 to 10 amino acid residues that are present in each variable domain. These six CDR loops (3 in the V_H and 3 in the V_L) provide the antigen-binding diversity of antibodies, determining the specificity and affinity for their respective antigens. CDRs 1 and 2 are encoded by the V gene segment of heavy and light chains, while the CDR3 is encoded by the junction of the recombined VDJ (heavy chain) and VJ (light chain) genes and is thus the most variable. Each light chain consists of a single variable domain and a single constant domain while each heavy chain consists of a single variable domain and either three or four constant domains, depending on the class of antibody. There are five classes of antibodies in mammals: IgA, IgD, IgE, IgG and IgM, each encoded by their respective heavy chain constant region genes, α, δ, ε, γ, and μ (119, 187). During the initial stages of the development of B cells in the marrow, two heavy chains consisting of the recombined VDJ and the μ constant domain associates with two VJ recombined light chains on the cell surface to form a membrane bound IgM. This complex along with a cytosolic heterodimeric signaling complex made up of CD79a (Ig-α) and CD79b (Ig-β) forms the BCR complex in immature B cells (133). At the end of the maturation process in the bone marrow, B cells expressing both membrane bound IgM and IgD migrate from the marrow as naïve B cells to the peripheral lymphoid organs (e.g. lymph nodes) where they mature further (36). Upon binding and activation of the B cell by its specific antigen, it proliferates rapidly, begins to make soluble antibodies and differentiates into an antibody-secreting plasma cell. During the replication process, IgH and IgL loci also undergo a process known as somatic hypermutation in which a high rate of point mutations generates

further diversity in the variable domains of the antibody chains (119). The B cells then undergo clonal selection and expansion based on antigen binding of those antibody mutants that have enhanced affinity for the antigens (i.e. affinity maturation).

Another process that occurs after B cell activation is isotype class switching, a further recombination process in the heavy chain loci occuring at the constant gene segments. As indicated, there are various classes of antibodies produced by B cells. While naïve B cells only produce membrane bound IgM and IgD, activated B cells secrete IgM, IgA, IgE or IgG antibodies. During the maturation of the B cell, DNA encoding the VDJ recombined variable domain of the heavy chains can undergo a downstream class switch recombination involving the constant γ, α or ε segments, resulting in a gene that now encodes for a different isotype, i.e. IgG, IgA or IgE respectively. This process preserves the antigen specificity of the antibodies but varies the effector function of the antibodies due to differences in Fc regions; the process is influenced by cytokines produced by neighbouring T cells in the lymphoid organs (157).

T cells and T cell receptors

T lymphocytes are the main effectors of cellular immune responses of the adaptive immune system (152). There are two major classes of T cells that are distinguished by the co-receptor expressed on the cell surface. Helper T cells express CD4 and cytotoxic T cells (also known as CTLs, cytotoxic T lymphocytes) express CD8. Helper T cells consist of a diverse group of $CD4^+$ T cells that play distinct roles by secreting a diverse collection of cytokines, depending on the lineage. The classical Th_1/Th_2 dichotomy established in the late 80s (137) has now grown to include Th_{17} cells,

natural regulatory T cells (nT_{reg}, developed in the thymus), induced regulatory T cells (iT_{reg}), T follicular helper cells (T_{fh}), Th_{22} and Th_9 cells (213, 229). The differentiation of naïve $CD4^+$ T cells is highly dependent on the cytokine milieu present during $CD4^+$ T cell activation. Activation results in the upregulation of distinct transcription factors and subsequent gene expression profile associated with the various lineages. For example, Interleukin-12 (IL-12) and Interferon-gamma (IFN-γ) are important for Th_1 differentiation, IL-2 and IL-4 are important for Th_2 cells, and, transforming growth factor β (TGF-β) and IL-6 are important for Th_{17} cells. The subsequent upregulation of the transcription factors T-bet, GATA-3 and RORγt results in the differentiation of the naïve $CD4^+$ T cell into the Th_1, Th_2 and Th_{17} lineages, respectively (213).

There are also a unique group of $CD4^+$ T cells known as the regulatory T cells (T_{reg}). These T cells operate to suppress immune responses of other cells, providing a self-checking system to prevent excessive responses that can lead to various diseases such as autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and also immune mediated pathogenesis due to elevated levels of inflammation.

The helper $CD4^+$ T cell lineages perform diverse functions in the overall immune response. Th_2 cells secret IL-4 and IL-5, cytokines that stimulate B cell proliferation and promote the class switching process in antibody production, and IL-13 that induced IgE class switching and also acts on epithelial and smooth muscle cells in the lung or intestinal compartments to increase mucus production, goblet cell metaplasia and airway hyper-responsiveness (AHR) (146). Thus Th_2 mediated immune responses are commonly associated with immunity against helminths and allergens resulting in

inflammatory diseases such as asthma or type I hypersensitivity. Th_1 cells on the other hand are associated with immune responses leading to delayed-type or type IV hypersensitivity (DTH) and protection against intracellular pathogens such as viruses (1). The primary cytokine secreted by Th_1 cells, IFN-γ, is the only type II interferon, and is a potent factor for the activation of macrophages, induction of IgG2a antibody class switching in B cells and regulation of numerous genes that are involved in antigen presentation pathways and antiviral immunity (26, 177).

The $CD8^+$ cytotoxic T cells (CTL), in addition to Th_1 cells, are involved in immune responses against intracellular pathogens (91, 223). $CD8^+$ T cells produce antiviral cytokines such as IFN-γ but they also offer protection directly killing the host cells (called target cells) that are required for pathogen replication. Target cell lysis is accomplished by inducing apoptosis in the target cell via two different mechanisms (182). One involves a membrane bound protein known as the Fas ligand, which binds and clusters the death receptor Fas expressed on the surface of the target cells. The adaptor Fas-associated protein with Death Domain (FADD) is then recruited to the cytosolic death domain of the Fas receptors; these in turn recruit the zymogen form of caspase-8 through its death effector domain (DED), forming the death-inducing signalling complex (DISC). Clustering of the pro-caspase-8 proteins results in their activation by autoproteolysis and the active caspase-8 goes on to cleave and activate effector caspases such as caspase-3, leading to apoptosis (111). Another mechanism by which cytotoxic T cells induce the death of their target cells involves the release of intracellular granules that contain cytotoxic machinery. These secretory granules contain perforin, a protein that polymerizes in target cell membranes to form transmembrane channels

analogous to those formed by complement proteins. The granules also contain serine proteases such as granzyme B that enter the target cells through the perforin channels, cleave and activate pro-caspases in the target cell, and thus initiate apoptosis (121).

Regardless of the function of the T cell, specificity is determined by the membrane-bound T cell receptor. Similar to the BCR on B cells, each T cell expresses a single variant of the TCR. However T cells are activated by a particular group of cells known as the antigen presenting cells (APCs) in the peripheral lymphoid organs. Most importantly, they also only recognize antigens in the form of short peptide fragments that are presented by surface molecules encoded by the major histocompatibility complex (MHC) (55). Just as there are two major classes of T cells ($CD8^+$ and $CD4^+$T cells), there are also two major classes of MHC products, class I and class II. The co-receptors CD4 and CD8 not only define the T cell classes, but they function by binding to class II and class I MHC products, respectively. This binding facilitates both the specificity and sensitivity of the T cells, in part by binding the corresponding peptide-MHC complex (pMHC) cooperatively with the TCRs (110), in the formation of what has been termed the “immunological synapse”.

The TCR is a heterodimeric receptor consisting of an α chain and a β chain, each made up of a single variable and a single constant immunologobulin-like domain (Note: there is also a $\gamma\delta$ heterodimer that is found on a smaller fraction of T cells) (15, 77, 129). Similar to the light chain and heavy chains of the antibody, the alpha and beta chains of the TCR are encoded by multiple gene segments: V, J, C segments for the alpha chain, and V, D, J and C segments for the beta chain (114). Diversity of the TCR is generated by the same RAG mediated recombination process as antibodies, during development

of the T cell in the thymus (174). During thymic development, T cells undergo rigorous selection to ensure that the TCR on a mature T cell is both MHC-restricted yet not overtly self-reactive. After recombination of the TCR genes, immature thymic T cells express both CD4 and CD8 (double positive), along with their unique TCRs. They undergo two selection processes. The double positive T cells first undergo “positive selection” in which only those cells that express a TCR that binds to self-peptide MHC complexes expressed by cortical epithelial cells in the thymus receive a survival signal. T cells that bind to either class I or class II pMHCs develop into $CD8^+CD4^-$ or $CD4^+CD8^-$ T cells, respectively (193). Cells that fail to express a functional, MHC-binding TCR undergo apoptosis.

The surviving T cells are further screened for self-reactivity in the medulla where APCs such as dendritic cells and thymic epithelial cells will present a repertoire of self-peptides to the maturing T cells, with the help of the transcription factor autoimmune regulator (AIRE) (145). At this stage, T cells that bind too strongly to these cells via a TCR-pMHC interaction will die by apoptosis and undergo deletion in a process known as “negative selection” or tolerance induction. Thus, mature T cells that migrate from the thymus have undergone selection for those TCRs that have a low affinity for self-peptide MHC, and against those TCRs that have too high of an affinity for self-peptide MHC.

Antigen Processing and Presentation

Proteins encoded by the major histocompatibility complex (MHC) are cell surface molecules that bind short peptide fragments and “present” them to T cells. The peptide-

MHC complex is recognized by a T cell receptor. As indicated, there are two major classes of MHC molecules, class I recognized by CD8 and class II recognized by CD4 (142). MHC class I molecules are dimers composed of a membrane bound α chain (also known as the heavy chain) and a smaller non-covalently associated β2-microglobulin molecule. MHC class II molecules are dimers composed of two similarly sized membrane bound α and β chains. Both class I and class II molecules form similar three-dimensional structures with four domains each. The peptide binding cleft is formed by two parallel alpha helices lying above a beta sheet; the two helices are formed by domains α1 and α2 of the MHC class I molecule and by domains α1 and β1 of the MHC class II molecule (122). The primary difference between the peptide-binding clefts of the class I and class II molecules is that the ends are more open in class II, allowing the peptide N- and C-termini to extend through and thus accommodating longer peptides. In contrast, in MHC class I molecules the N- and C-termini of the peptides are generally buried within the molecule, such that shorter peptides are present. Hence, there is a subsequent difference in the length of peptides bound in MHC class I and class II molecules. The peptides found in complex with class I MHCs are short, usually 8-10 peptides in length, whereas those bound in class II are longer with at least 13 amino acids. Also, as the ends of the peptides in the pMHC class I complex are buried in the molecule, conserved residues known as anchor residues make the main stabilizing interactions while the binding cleft in the MHC class II is more permissive (45, 65).

In humans, each individual inherits three MHC class I genes (HLA-A, -B and -C) coding for the heavy chain, and three MHC class II genes (HLA-DP, DR, DQ) coding for the α and β chains from each parent. β2-microglobulin is conserved and coded by a

gene that is not related to the MHC family of genes. Among the population, most of the MHC genes further exist in one of hundreds of possible alleles, making MHC genes both polygenic and highly polymorphic (97, 158). Polymorphisms in each allele include residues in the peptide binding pocket, hence each MHC protein would be able to present a different spectrum of peptides. These features allow a greater range of potential pathogenic antigens to be recognized by T cells. However, polymorphisms are also associated with either enhanced or reduced responsiveness to some pathogenic organisms, as these polymorphisms can affect binding to either self-peptides (and thus affect the processes of thymic selection) or they can influence the binding of the pathogenic peptides. For example, recent studies of HIV have shown that there is an association between elite responders and MHC class I polymorphisms (39).

The peptides presented by the MHC class II and I molecules differ in their intracellular processing pathways (81). MHC class II molecules present peptides that originate from endosomes and are expressed mainly by professional antigen-presenting cells. APCs such as macrophages and dendritic cells take up extracellular pathogens and antigens via phagocytosis or endocytosis. The pathogens are broken down and the proteins are then degraded into smaller fragments by acid proteases such as cathepsins that are present in the late endosome and lysosomes. Proteins that bind to the BCR on B cells are also internalized by receptor-mediated endocytosis and processed similarly. Newly synthesized MHC class II αβ dimers in the endoplasmic reticulum (ER) are associated with an invariant chain to stabilize the molecule and to prevent the class II molecule from binding any peptides found in the ER. The invariant chain also targets the delivery of the class II molecule to the endosomal compartment

where acid proteases will cleave the invariant chain, leaving a short fragment called CLIP (class II-associated invariant chain peptide) still bound in the binding site of the class II molecule. Pathogenic peptides are still unable to bind to the class II molecule due to the presence of CLIP until a MHC class II-like molecule known as HLA-DM promotes the dissociation of CLIP and stabilizes the empty class II molecule until an antigenic peptide is loaded into the binding cleft. Another function of the HLA-DM is also to remove weakly binding antigens in a process known as peptide editing (7), ensuring only the presentation of stable antigen peptide MHC complexes. Finally, the stable complex is transported to the surface of the cell, where it can stimulate $CD4^+$ T cells.

The MHC class I molecule is expressed by all nucleated cells. These molecules express either self peptides or antigen fragments, e.g. derived from viral proteins that are synthesized in infected cells. Cytosolic proteins, including the viral and self-proteins, and misfolded proteins in the ER are continuously degraded by the proteasome (92). The peptide fragments formed are then delivered into the ER as potential ligands for the MHC class I molecule by ATP-binding cassette (ABC) proteins in ER membrane known as ‘transporters associated with antigen processing-1 and -2’ (TAP-1 and TAP-2). The newly synthesized class I heavy chain is refolded with the help of the chaperon protein calnexin, followed by calreticulin. The refolded heavy chain then associates with β2-microglobulin and along with tapasin and Erp57, forms the peptide-loading complex in the lumen of the ER. The TAP complex formed by TAP-1 and TAP-2 then associate with this peptide-loading complex and transport peptides from the cytosol to the proximity of the empty MHC class I molecule. In an uninfected cell, self peptides are loaded onto the MHC class I molecule and the peptide-loading complex dissociates,

allowing the refolded MHC molecule to be transported out of the ER to the cell surface. However, if the cell is infected by a virus, peptides derived from viral proteins that are being produced in high quantity for the replication of the virus can be loaded into the binding cleft of the MHC class I molecule and presented on the cell surface for recognition by a $CD8^+$ T cell, leading to destruction of the infected cell.

The Hepatitis B Virus

Hepatitis B virus (HBV) is a non-cytopathic, hepatotropic member of the hepadnaviridae family that infects only humans and chimpanzees. Other related viruses infecting ground squirrels, woodchucks and waterfowl such as geese and ducks have also been found. HBV infection in most adults is acute, and ultimately viral clearance is achieved. However, an estimated 350 million people in the world are chronically infected with HBV, leading to almost a million deaths a year from HBV related complications. A highly effective vaccine (Recombinvax HB®) consisting of recombinant surface antigen (HBsAg) produced in yeast cells has been developed (205) and has been successful in preventing new infections in countries that introduced vaccination programs. Unfortunately, the prophylactic vaccine produced only transient anti-viral effects in chronic patients, with limited effective control of the infection. HBV causes an inflammatory state in the liver and failure to clear the virus leads to chronicity, liver cirrhosis, and eventually hepatocellular carcinoma (HCC). Although the hepatitis B virus does not directly cause the cellular death associated with liver diseases, pathogenesis is attributed to the corresponding immune response (46, 68, 86). In acute patients, a robust immune response successfully eradicates the viral infection with minimal injury or

lasting effects on the liver. However in chronic patients, a poor immune response allows the virus to persist and sustained liver disease with associated liver cell destruction and regeneration, and inflammation. Thus the chronic liver cell injury presents a potential oncogenic state; after a long period of time there is increased probability of the accumulation of genetic aberrations, random viral integration into the genome, and other viral factors that affect cellular pathways, eventually contributing to the development of HCC.

HBV biology and antigens

The hepatitis B virus is an enveloped virus with a partially double-stranded, relaxed circular DNA (rcDNA) genome of 3.2kb that consist of a complete minus strand and a partially synthesized plus strand (Figure 1.2) (179, 198). Viral reverse transcriptase is attached at the 5' end of the minus strand and a short RNA is attached to the 5' end of the plus strand. During infection, the reverse transcriptase and RNA fragment are removed and the rcDNA is converted into a covalently closed circular DNA (cccDNA). This cccDNA then serves as the template for the transcription of four mRNAs that encode for the core and envelope structural proteins and the non-structural proteins pre-core, polymerase and X proteins. The largest 3.5kb mRNA is greater than the HBV genome itself and serves as the pre-genomic RNA that is packaged into viral capsids and used as a template for the replication of the HBV DNA genome. The pregenomic mRNA also contains three open reading frames that code for the core, pre-core and polymerase proteins. The core proteins, also known as the hepatitis B core antigen (HBcAg), are main structural proteins that form the viral capsids. The pre-core protein, a

longer version of the core protein, with an extended N-terminal sequence coding for a signal peptide, does not play a part in the viral particle formation but is instead translocated to the ER where further processing results in secretion of the protein as an extracellular form of the HBcAg known as the HBeAg. The polymerase protein is translated from an internal ribosomal initiation site in the pregenomic mRNA. The multifunctional polymerase protein is not only a reverse transcriptase, but also a DNA polymerase, and an RNAase, and it contains an amino-terminal domain that functions as a protein-primer during reverse transcription of the viral pregenome.

The envelope consists of three structural proteins encoded in the envelope open reading frame (ORF). These three overlapping proteins called small (S), medium (M) and large (L) are translated from three different start codons in the ORF and contain the hepatitis B surface antigen. In addition to the S protein, the additional domain in the M protein is called the pre-S2 domain, while the L protein contains this and an extra pre-S1 domain. All three proteins are essential components of the HBV virion (also known as the Dane's particle) and are transmembrane proteins that form multimers via disulphide bridges between cysteines in the S domains (202). Interestingly, smaller non-infectious particles containing predominantly the S and M proteins are made in excess of Dane particles, accumulating in the blood of infected patients. On the other hand, the excess L proteins form long filamentous particles that are not secreted but are accumulated in the ER causing the ER to be enlarged, giving the infected hepatocyte the histologically characteristic "ground glass" appearance (210). The pre-S1 domain of the L protein functions as a ligand for the binding of the core proteins in the capsids prior to the assembly of the viral particle that eventually buds off from the ER membrane.

The pre-S1 domain of the L proteins is found in the cytosolic side of the viral membrane to interact with the core proteins during viral particle assembly (147). A conformational change in the protein causes pre-S1 domain to be displayed outside the viral particle. While the route of infection of the HBV virus is still unknown, there is evidence that the translocated pre-S1 domain of the L protein is an important substrate for the viral receptor as neutralizing antibodies against epitopes in the pre-S1 domain are able to attenuate viral replication (179). Although numerous studies have suggested that cell membrane proteins such as annexin V, apolipoprotein H and the transferrin receptor liver all interact with HBV envelope proteins, it has not been demonstrated that they are involved in the infection of hepatocytes (86).

The fourth open reading frame in the HBV genome codes for a transcriptional transactivating protein known as the X protein (HBx) (179, 224). In vitro studies have shown that the X protein is able to activate the transcription of numerous host genes and also viral genes, stimulate signal transduction pathways and bind various host proteins. However, the overall physiological function of X is still not very clear although it has been shown to be essential for infection in vivo but not in vitro.

Immune responses to HBV infection

Virus infections usually induce an innate immune response involving the production of type I interferons (IFN-α and -β), leading to the upregulation of genes that promote an environment inhibiting the replication of virus (171). However, hepatitis B virus infections in animal models have showed that the HBV virus can elude innate immune detection systems such as the Toll-like receptors, preventing induction of IFN-α/β (218). The kinetics of HBV replication in the body provides a possible explanation. It

has been observed that HBV replication has an initial lag phase of 4-7 weeks, during which viral DNA and antigens are not detectable in serum or the liver; after this, the virus undergoes logarithmic replication in which most hepatocytes are infected (219). Since innate immune responses were not detected during the lag period, the absence of HBV replication does not appear to be a consequence of active immune suppression by the virus. Another possible strategy adopted by the virus to evade the innate immune system is to retain its transcriptional template in the nucleus, away from the innate sensing molecules. In addition, the viral mRNA resembles host cellular transcripts, with a 5' cap and 3' polyadenylation and also by encapsulating its replicating genome in protein capsid particles in the cytoplasm (179, 219).

The adaptive immune system plays the major role in controlling HBV infections (20, 21, 160). A robust immune response mounted by the adaptive immune system is associated with the clearance of HBV in acute infections whereas a weak and transient response is highly correlated with chronic infections (69, 104, 215). In particular, robust T cell responses, both $CD8^+$ and $CD4^+$ T cells are found in patients that resolve infection. Th_1 cytokines such as IFN-γ and TNF-α contribute to viral control but importantly, $CD4^+$ T helper cells appear to play an important role in inducing $CD8^+$ T cell responses against HBV infection; e.g. vigorous $CD4^+$ responses coincide with $CD8^+$ responses. Viral epitopes that trigger these immune responses have been well studied for both $CD4^+$ (67) and $CD8^+$ T cells (19, 141, 159, 185). More importantly, studies with MHC HLA-$A2^+$ patients have also allowed the identification of immunodominant epitopes; Core 18-27 (124) and Env 183-191(215) that make up the majority of $CD8^+$ immune responses.

In addition, while $CD8^+$ T cells aid in the control of HBV by eliminating infected hepatocytes, B cells provide humoral immunity against HBV infection mainly by secreting antiviral antibodies to neutralize viral particles. Detection of antibodies against the various hepatitis antigens in the sera of patients is commonly used as one diagnostic marker for the stages in infection (160). Anti-HBcAg IgM is the first antibody to develop during an acute infection and HBcAg IgG is commonly found in circulation post infection; anti-HBeAg antibodies and anti-HBsAg antibodies develop later, and help in both resolution of the infection and providing protective immunity. The recombinant HBsAg vaccine stimulates the immune system to produce the neutralizing anti-HBsAg antibodies and have since been successful in reducing HBV incidence in the last 30 years (103).

Chronic HBV infections and hepatocellular carcinoma

Chronic infections in patients occur when immune responses of the adaptive immune system fail. While cytotoxic $CD8^+$ T cells in the control of HBV infection are without doubt important, the pathogenesis of liver disease during chronic infections can also be attributed to weak CTL responses (123). Factors contributing to the lack of a robust immune response are unclear but studies have shown that T-cell anergy could be due to high viral load. The viral precore (HBeAg) does not play a part in virus replication but may play a part in inhibiting the immune system. In transgenic mouse models, the HBeAg has been shown to anergize antigen specific T cells and suppress antibody production (40). In chronic patients with high levels of HBsAg in their sera, antigen specific T cell responses were weak and had altered pMHC binding (161).

Mutations in immunodominant viral antigens have also been found in chronic patients, allowing the mutant virus to evade immune surveillance or antagonize T cell activation (18, 22). Lastly, down regulation of MHC class I molecules had been observed in animal models (135) while the HBx protein has been shown to be able to inhibit processing by reducing the proteasome activity when overexpressed (98). A reduction in MHC expression and processing capacity would further allow the virus to elude immune detection.

Although the hepatitis B virus is non-cytopathic, cytotoxic T cells that recognize infected hepatocytes rapidly induce them to undergo apoptosis. Secretion of chemokines such as CXCL9 and CXCL10 induced by IFN-γ results in the recruitment of inflammatory cells, including virus-nonspecific cells, to the liver (106). These non-specific immune cells contribute to the necroinflammatory state of the infected liver by further secretion of inflammatory cytokines and hepatocytes death. Liver cells are terminally differentiated cells with a unique regenerative capacity in which healthy hepatocytes rapidly divide to replace destroyed cells in response to liver injury. Thus, in an acute infection, infected hepatocytes are successfully eliminated and hepatocellular regeneration ensures that the organ is fully recovered and the reparative processes are turned off when viral clearance that been achieved. However, when the immune response is inefficient in eradicating the virus in chronic patients, persistent inflammation in the liver results in continuous low-level destruction of hepatocytes that accumulates into fibrosis, cirrhosis and eventually leads to the development of hepatocellular carcinoma (86).

Engineering TCRs and Antibodies Against HBV pMHCs

Products of the major histocompatibility complex present peptides to T cells to facilitate antigen specific immune responses. The peptides presented by MHC class I proteins derived from cytosolic proteins may originate from viral proteins but also self-proteins leading to the discovery of tumor associated antigens (28, 168) that are presented by MHC proteins on the surface of tumor cells. The use of soluble multimeric pMHC complexes, described first in 1996 by Altman *et al* (6), contributed significantly to the study of specific T cell populations. Conversely, there are few tools to study specific pMHC complexes on the surface virally infected or tumor cells. Soluble forms of the T cell receptor would be the most obvious solution but the low affinity and stability of TCRs as recombinant soluble molecules has made it difficult to use them as detection tools (9, 169),

Monoclonal antibodies with TCR-like specificities also offer a possible alternative (139, 149). The ability to have tools that target specific pMHC complexes would enable the study of the expression and distribution of these complexes on the surface of tumor cells and virally-infected cells. Visualization and quantification of specific pMHC complexes during diagnosis of patients would also aid in deciding the appropriate therapeutic measures of the best efficiency, especially in the administration of TCR gene transfer therapy (178). Furthermore, with the ability to target specific pMHC presented on tumor or virus-infected cells, directed delivery of drugs, immunotoxins, cytokines or therapeutic molecules, such as bi-specific antibody fusions could be enabled (13, 35, 63, 136, 216).

Soluble T cell receptors

The first structures of both a human and a mouse αβ TCR was published in 1996 (75, 77). Unlike antibodies, TCRs are membrane bound receptors with a hydrophobic transmembrane domain; thus, only the extracellular domains of the individual TCR chains were expressed and crystalized. Several groups had introduced additional stabilization domains such as *jun/fos* leucine zippers to aid in the refolding of the heterodimer (89, 221). Further engineering to increase the stability of the recombinant TCR have included the introduction of non-native disulfide bonds to replace the native bond formed by cysteine residues found in the stalk domain of the α and β chains that is excluded in the truncated construct. One strategy adopted was the use of C terminal extensions to the constant domains of the individual chains (188) while another group introduced the cysteine residues through rational design in the constant domains of the α and β chains (30). Mutagenesis at position 48 of the α-constant domain and 57 of the β-constant domain was done to introduce two cysteine residues that would be positioned in close proximity based on available structure of the constant domains, allowing a disulfide bond to be formed. Various eukaryotic expression systems such as insect cells and Chinese hamster ovary cells have been used with some success (8, 77, 89, 211). However, some soluble TCRs were in an *Escherichia coli* expression system and refolded from inclusion bodies, with the benefits of higher yields with lower costs (30, 56, 75, 188, 207, 220).

T cell receptors have low affinities, with K_d values in the range of 1-100μM for their respective pMHC ligands, and with binding half-lives of only a few seconds (55). While B cell-derived antibody genes undergo somatic hypermutation after B cell

activation leading to the generation of mutants with enhanced affinity for the antigen. TCR genes do not undergo somatic hypermutation (71). Furthermore, during development in the thymus, only TCRs with very low affinity for self peptide-MHC are selected after rounds of positive and negative selections. One strategy used in an attempt to overcome the low affinity involved TCR multimers (115, 190), analogous to soluble pMHC tetramers (6). Laugel *et al* showed that the increased avidity was able to allow the HTLV-1 transcription factor Tax peptide specific A6 TCR and the influenza A matrix antigen specific JM22 TCR to exhibit 1339- and 444-fold slower dissociation rate than their monomeric interactions respectively. The increased avidity in these tetrameric molecules, and higher order oligomers using quantum dot scaffolds, allowed them to be used to stain cells (9, 115, 190). However, an important caveat to the use of such multimeric molecule is that the avidity benefits may be negligible with low physiological surface antigen densities (9). Another solution to the low natural affinity of TCR for their pMHC ligands is the enhancement of monomeric affinity by in vitro protein engineering, using the process of directed evolution. Mutant libraries are generated and screened for higher affinity variants using various techniques such as phage display (118), yeast surface display (47, 95, 108) and mammalian cell display (41). This strategy generated TCRs with improved affinities in the range of picomolar to nanomolar, allowing cell staining with monomeric TCR molecules.

TCR-Like antibodies

Antibodies with TCR-like specificities have been generated by several groups as tools to study antigen presentation, for directed delivery of payloads, or as specific

inhibitors of T cell activation (113, 138, 148, 151, 162). Although there has been some success with conventional methods, i.e. hybridoma or immunization techniques, to generate antibodies with TCR-like specificities, such antibodies are still rare as B cells, unlike T cells, do not require MHC-restriction in their specificities (i.e. most of the antibodies are not directed at the peptide, but bind to the MHC alone). However, the use of large phage-display libraries has allowed the isolation of antibodies specific for HLA-A2 MHC class I molecules restricted by tumor associated epitopes, such as the telomerase catalytic subunit (hTERT) epitope (117), the melanoma differentiation antigen gp100 (57) and viral epitopes such as the human T-cell lymphotrophic virus-1 (HTLV-1) transcription factor Tax (49) and influenza matrix protein M1 (23). The molecules have since been used to visualize and quantify specific pMHCs on transfected cells, tumor cells, virally infected cells and antigen presenting cells. Interestingly, studies with TCR-like antibodies structures and comparisons between TCRs and TCR-like antibodies specific for the same pMHC ligand had shown that while these antibodies exhibit TCR-like specificity, they do not necessarily recognize the same contacts on the pMHC ligand (23, 126, 127, 134). Furthermore, they also show that unlike TCRs (12, 170), TCR-like antibodies do not have a conserved binding footprint on the pMHC ligand.

In vitro Engineering of T Cell Receptors

The ability to engineer T cell receptors in vitro has allowed the production of stable soluble TCRs with enhanced binding affinities for their pMHC ligands (164). In addition, the use of adoptive T cell therapy with T cells of redirected antigen specificities

is a fast developing field (37, 48, 51, 178, 225). Co-receptors, i.e. CD4 and CD8 in helper and cytotoxic T cells respectively, aid in the binding of pMHC ligands and activation of the T cells (208). Thus, T cells transduced with genes of a normal, wild-type TCR typically still require the appropriate co-receptors for activation. However, studies have shown that T cells transduced with engineered class I restricted TCRs above a particular affinity threshold (K_d values below 1 μM) exhibit co-receptor (CD8) independence (42, 43). This expands the application of TCR gene transfer by allowing the re-directed helper activities of $CD4^+$ T cells to MHC class I restricted antigens. Thus engineering of T cell receptors for enhanced affinity will not only allow the use of soluble TCRs as tools for detection or directed delivery of therapeutic payloads, they could potentially expand the use of the receptors in adoptive T cell therapy.

The general strategy adopted for T cell engineering involves three steps; first, the identification and cloning of the α and β genes from a T cell clone with the desired specificity; second the generation and expression of mutant variants of the TCR in a display system; and finally the systematic, high-throughput screening of the TCR mutants for improved binding. As with soluble TCR expression, success in TCR engineering has been limited in part due to the relative instability of the TCR α and β chains, compared to the heavy and light chains of antibodies, such that not all TCRs are displayed efficiently. Nonetheless, phage, yeast and mammalian display systems have enabled the isolation of TCRs with enhanced affinity (41, 47, 94, 95, 118, 214).

Yeast display engineering

In the yeast display system, proteins of interest can be expressed as a fusion with the yeast mating protein Aga2p (25), allowing the proteins of interest to be localized on the surface of the yeast cells to facilitate subsequent analysis and screening. Gene sequences of mutants isolated after screening can be easily obtained by recovering plasmids, and sequencing. Yeast display has been successful in the engineering and isolation of lead antibodies (33, 66, 153). The first engineered TCR with over 100-fold improved affinity for the cognate pepMHC ligand was the mouse 2C TCR, engineered by yeast display (95). Along with the 2C TCR, the mouse class II MHC-specific TCR 3.L2 and TCR Vβ domains (34, 59, 109, 212, 214) have all been engineered using yeast display for improved binding to their cognate pMHC ligand or superantigen, respectively. TCRs are heterodimeric molecules consisting of the α and β chains. However to avoid the difficulties in maintaining stable expressions of two separate chains in yeast cells, the 2C and 3.L2 TCRs were engineered by yeast display as a "single-chain" construct whereby only the variable domains of the α and β chains were expressed, with a peptide linker connecting the domains. However with the truncation of the TCR, the single-chain constructs and the Vβ domains were inherently unstable; to "stabilize" the domains, the genes were subjected to random mutagenesis across the entire construct to generate mutant libraries that could be screened by high-speed cell sorting for surface expression. Mutants with higher levels of surface expression were also capable of being expressed at higher levels in various expression systems, including *E. coli,* allowing the production of these soluble proteins for further studies and applications such as therapeutics or targeted delivery.

Figures

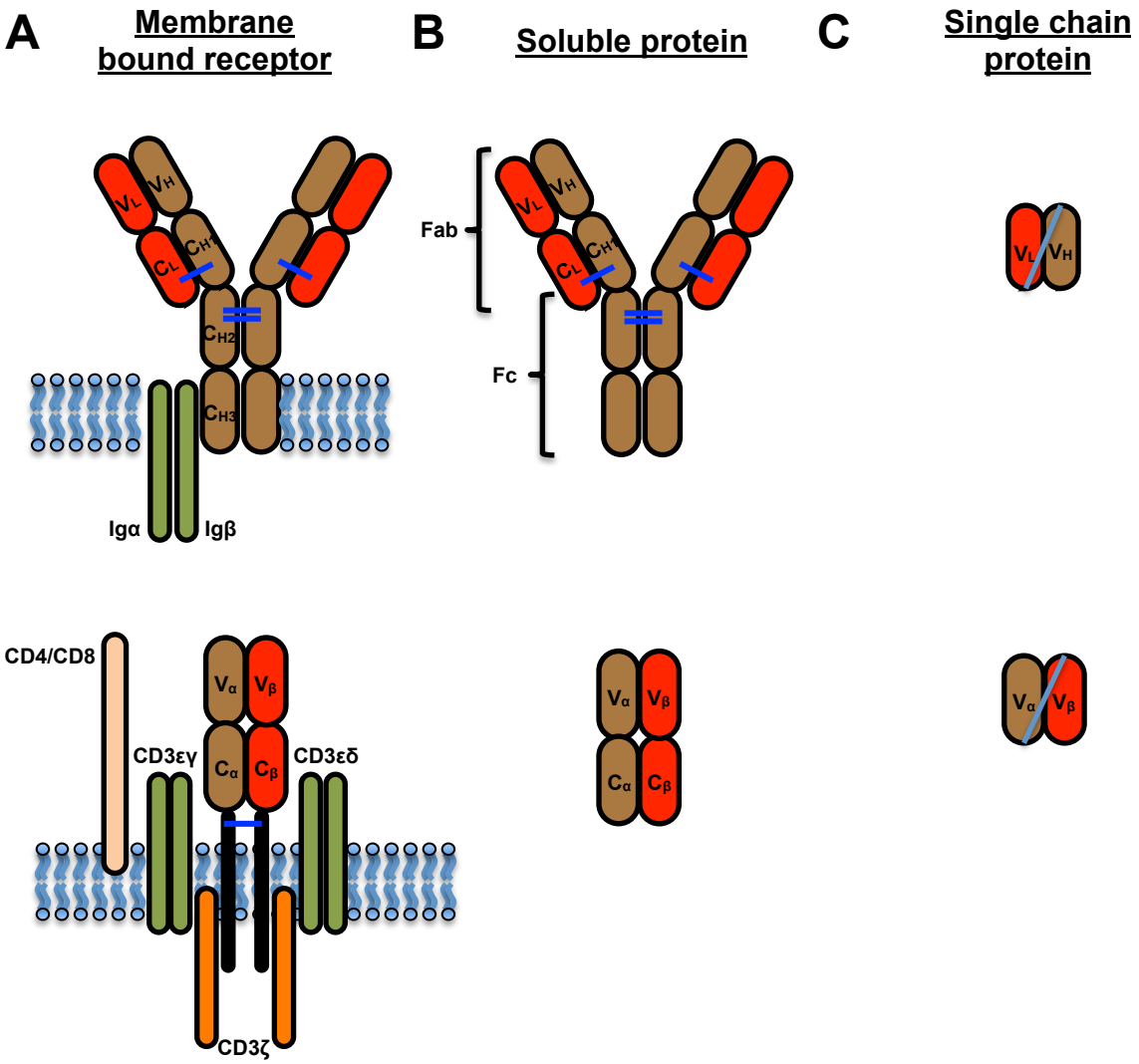


Figure 1.1. Immune receptors of the B cell and the T cell. (A) The B cell receptor consist of an membrane bound IgM associated with the Igα and Igβ signaling proteins. The T cell receptor is a heterodimeric protein consisting of the α chain and the β chain each made up of a single variable (V) and a single constrant (C) domain. The complex also consist of the CD3 protein made up of γ, δ, ε and ζ chains, and the CD4 or CD8 co-receptor. (B) The antibody is the soluble form of the BCR while TCRs are recombinantly expressed as a truncated receptor in soluble form. (C) Engineered single chain versions of the receptors consist of just the antigen specific domains fused via a flexible linker.

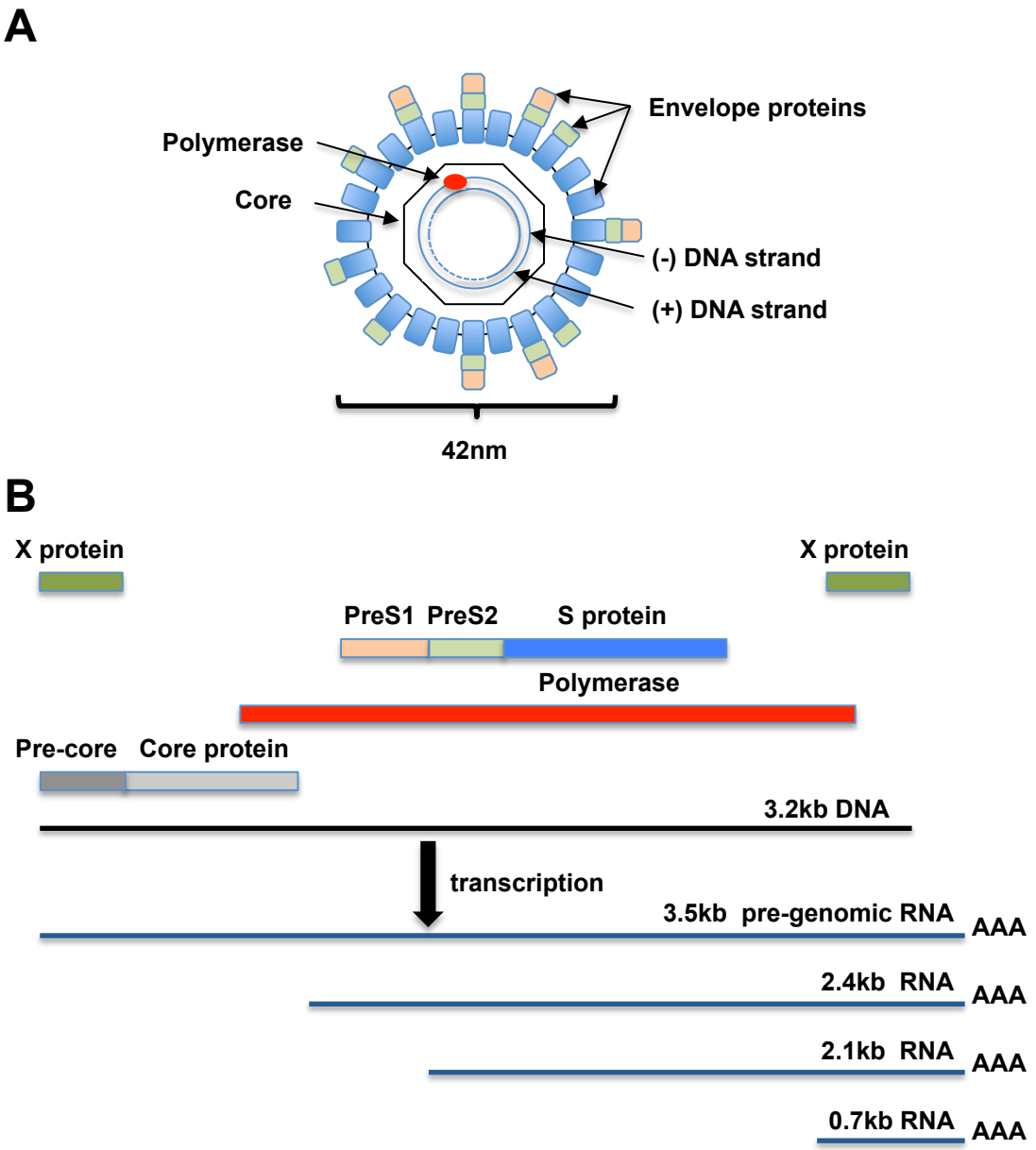


Figure 1.2. Structure of hepatitis B virion and genome. (A) Diagram of HBV virion. The virion has a diameter of about 42nm and consist of an icosahedral core encapsulating the HBV DNA genome and the polymerase protein, and an outer lipid envelope embedded with the S, M and L proteins. (B) Genomic organization of HBV. 4 mRNA transcripts are produced, the 3.5kb pre-genomic RNA that also contains the ORF for the pre-core, core and polymerase proteins; the 2.4kb and the 2.1kb mRNAs that produces the S, M and L proteins and the 0.7kb mRNA that is translated into the X protein

CHAPTER TWO

CHARACTERIZATION OF T CELL RECEPTOR-LIKE ANTIBODIES SPECIFIC FOR HEPATITIS B ANTIGENS

Introduction

The complex between a viral peptide and a class I product of the major histocompatibility complex (pMHC) is a cell surface marker of an infected cell. Viral proteins produced in the infected cell are degraded by the proteasome, the resultant peptides are loaded onto MHC class I molecules in the endoplasmic reticulum, and the pMHC is transported to the plasma membrane for extracellular presentation (92). $CD8^+$ T cells expressing a specific T cell receptor are able to recognize the viral pMHC. The binding process leads to activation of the T cell, resulting in the lysis of the infected cell and control of viral replication. The quantity and density of the specific pMHC molecules are factors that influence the immune response (105). Thus, the ability to examine the presence and distribution of specific viral pMHC would benefit our understanding of viral infections and could inform decisions on anti-viral therapies.

Class I MHC molecules bind peptides of 8 to 10 amino acids in length. As virus genomes encode proteins that consist of a few thousand to tens of thousands of amino acids in total, the number of possible peptide sequences presented by the MHC class I molecule of the infected cell can be large. Yet interestingly, the number of immunogenic peptide sequences is small in comparison. This is partly due to the restrictions imposed by the nature of the MHC class I molecules themselves, as they require the peptides to have particular “anchor” residues for efficient binding. Among the immunogenic

peptides, immunodominant epitopes are defined as peptides recognized by the most abundant T cell populations. Discovery and characterization of such peptides are areas of interest in viral infection research, such as with the hepatitis B virus, as they provide information about possible vaccines or other immune-based therapies. Two such immunodominant hepatitis B virus peptides are the $Core_{18-27}$ and $Env_{183-191}$ peptides, which are restricted by the HLA-A2 molecule, the most common class I allele in the human population. T cell clones specific for these pMHCs have also been isolated from patients. As the most abundant epitopes would also be the most desirable target for directed therapy, additional probes to study the distribution of these pMHC molecules would be useful.

Quantification of endogenously formed pMHC class I complexes has been achieved through various approaches (226). One approach correlates the amount of synthetic peptide required for T cell activity in comparison to activity with the infected cells; this approach is based on the assumption that the synthetic peptide is the same as the endogenously processed peptide. Also, the peptides (synthetic and endogenous) are assumed to be presented in the same conformations by the MHC moelcules, although it has been shown in a MHC class II system that differences can occur (150).

Another more tedious approach involves elution of the peptides from the pMHC complexes of the infected cells, and purification of the peptides by chromatography; the purification and yields are followed by T cell activity assays, in comparison with a defined synthetic peptide (64, 90). This process is often unsuccessful due to the low levels of the endogenous peptide, compounded by the inconsistency and inefficiency of the peptide extraction process, thus limiting the practicality of this technique.

Finally, more recent approaches have attempted to generate soluble binding probes with specificity for the pMHC, using the probes to detect the pMHC *in situ*. Accordingly, with the use of fluorescence-based detection techniques such as flow cytometry and microscopy, pMHC can be directly quantified on the cell membrane without complicated processing.

The natural receptor for the pMHC is the heterodimeric membrane bound T cell receptor (TCR). However, the inherent instability of these molecules in soluble form, and their low binding affinities for the pMHC, have complicated their use as a probe for detection (9, 169). Antibodies, on the other hand, can provide a source of soluble molecules with high affinities and specificities that have been used routinely in numerous research applications. Antibodies with TCR-like specificities have recently been isolated or engineered against numerous tumour-associated and viral epitopes (23, 57, 113, 117, 134, 139, 149), but to date they have not been generated against HBV related peptides. In this chapter, I describe the characterization of two monoclonal antibodies that have been isolated with TCR-like specificities for the two different HBV immunodominant epitopes, the $Core_{18\text{-}27}$ (Core18/A2) and $Env_{183\text{-}191}$ (Env183/A2) peptides restricted by the HLA-A2 class I molecule. I show that the antibodies are highly specific for their respective peptides in complex with the HLA-A2 class I molecules, specifically the HLA*A02:01 and HLA*A02:02 alleles and compared the fine specificity of the antibodies with the T cell clones that are specific for the same pMHCs. I also demonstrated several applications of the antibodies in the study of the quantity and distribution of the pMHCs. The antibodies were used to visualize pMHCs on the surface of cells and liver biopsy sections by microscopy. The antibodies were used to quantify

pMHCs on the surface of antigen presenting cells and its effect on the activation of T cell clones with the same specificities. Lastly, I utilized the C18/A2 antibody to examine the effect of interferons α and γ on the number of specific pMHC presented on the surface of HBV infected cells.

Materials and Methods

Generation of TCR-like antibodies

The TCR-like antibodies were previously generated by Konduru S.R. Sastry. In brief, inclusion bodies of the HLA-A201 MHC class I heavy chain and β_2-microglobulin were expressed in *Escherichia coli* cells and refolded *in vitro* in the presence of excess peptides (HBV $Core_{18-27}$ or $Env_{183-191}$). Purification of refolded complexes was done using ion-exchange and size exclusion column chromatography and used in the immunization of BALB/C mice in a regime consisting 4 doses at 2-week intervals by interperitoneal injection of solutions consisting of 25μg of purified pMHC monomer and Freund's complete adjuvant (primary dose) or incomplete adjuvant (booster doses). Isolated splenocytes from immunized mice were fused with NS1 myeloma cells at a 1:1 ratio to generate hybridoma cells. Supernatants of hybridoma were then collected and tested for specific binding. T2 cells pulsed with 1μM HBV $Core_{18-27}$ or Env_{183-91} peptides were incubated with 50 μL of hybridoma supernatants and binding was detected with anti-mouse IgG Alexa Fluor 488 secondary antibodies. T2 cells pulsed with influenza M1 peptides were used as a control. Positively screened hybridoma cultures were subjected to rounds of limiting dilution and screening to identify single hybridoma clones.

Cell lines

T2 cells (ATCC CRL 1992) and EBV transformed B cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, 0.5 mM sodium pyruvate, minimal essential medium (MEM), nonessential amino acids, Glutamax, 5 µg/mL Plasmocin (InvivoGen), 100 U/mL penicillin, and 100 µg/mL streptomycin. HepG2 and HepG2 H1.3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 20 mM HEPES, 0.5 mM sodium pyruvate, minimal essential medium, nonessential amino acids, Glutamax, 5 µg/mL Plasmocin (InvivoGen), 100 U/mL penicillin, and 100 µg/mL streptomycin. To boost HBV production, HepG2 H1.3 cells were transferred to Williams E medium supplemented with 5% FBS and in addition to the ingredients described above, 22 µg/mL hydrocortisone, 7 µg/mL inosine, 10 µg/mL insulin and 1% dimethyl sulfoxide. HBV-expressing HepG2-117 cells were cultured in the same culture media as mentioned above plus HBV expression is suppressed by 1 µg/mL doxycycline in HepG2-117 cells. HBV expression was boosted with the withdrawal of doxycycline from culture media along with the addition of 1% DMSO and 1000U/mL of IFN-γ.

Detection of pMHC complexes on pulsed and HBV producing cells

The T2 cell line (ATCC CRL 1992) is a mutant cell line that lacks the transporter associated with antigen processing 1 (TAP1) and TAP2 genes, which allows the efficient loading of peptides onto nascent HLA chains while the HepG2 cell line is an HLA-A*0201^{+} hepatoma cell line (ATCC HB-8065). T2 and HepG2 cells were pulsed with peptides at the indicated concentrations at 4°C for 60mins before washing with

fluorescence-activated cell sorter (FACS) staining buffer (1% bovine serum albumin [BSA] in phosphate-buffered saline [PBS] with 0.01% sodium azide). Staining of the pulsed cells was done with the antibodies diluted in FACS buffer to the required concentrations in 50µL final volumes for at 4°C for 60mins. Cells were subsequently washed before incubating with 1µg/mL of fluorescence-conjugated anti-mouse IgG secondary antibody in 50 µL staining buffer at 4°C for 30mins. After further washing steps, cells were analysed with a BD FACSCanto (Becton, Dickinson and Co.) flow cytometer. Data acquired were further analysed by FlowJo (Tree Star).

Surface plasmon resonance

Binding studies were carried out using the BIAcore™ 2000 (BIAcore AB). HLA-A*0201 monomers presenting the $Env_{183-191}$ peptide and $Core_{18-27}$ were immobilized onto CM5 binding chips by amine coupling at pH 6.0. A titration of concentrations of the Core18/A2 and Env183/A2 antibodies were flowed over the pMHC ligands at a flow rate or 5µL/min. Measurements were done at 25°C in 20mM Tris pH 8.0. Kinetic binding parameters were determined using BIAevaluation software.

Quantification of pMHC complexes

T2 cells pulsed with a titration of HBV $Core_{18-27}$ or $Env_{183-191}$ peptides were stained with the respective antibodies with TCR-like specificities as described above. The QIFI™ Kit (Dako) consisting of a range of beads with known numbers of IgG complexes was used as a standard for comparison. Antibody stained T2 pulsed cells and the standard beads were stained with the same secondary antibody preparation

and analysed by flow cytometry. The number of pMHC complexes on the surface of the cells can be inferred by comparing the MFI observed against a standard curve generated from the MFI obtained from the analysis of the standard beads.

HBV-specific $CD8^+$ T cells degranulation assay

$CD8^+$ T cells specific for the $Core_{18-27}$ or $Env_{183-191}$ HLA-A2 pMHC cells (80) were incubated with peptide-pulsed T2 cells with phycoerythrin conjugated anti-CD107 antibody (BD Pharmingen) 37°C for 5 hours. Cells were then washed and stained with phycoerythrin-Cy7 conjugated anti-CD8 antibody at 4°C for 15mins before washing and analysis by flow cytometry.

Visualization of pMHC complexes by fluorescence and confocal microscopy

HepG2 and HepG2 H1.3 cells were cultured on coverslips in media. Coverslips were then washed with PBS and fixed with 1% paraformaldehyde (PFA) at room temperature for 15 mins. Coverslips were then washed thoroughly with PBS and blocked with 5% goat serum at room temperature for 60 mins. Staining of the cells were done with 10ug/mL of Core18/A2 specific antibody at 4°C overnight. Coverslips were then washed and incubated with anti-mouse IgG-horseradish peroxidase (HRP) polymer (Dako) before performing the tyramide signal amplification according to manufacturer's instructions using the Alexa Fluor 647-tyramide signal amplification kit (Invitrogen). Coverslips were then mounted using Antifade gold reagent containing DAPI (4′,6-diamidino-2-phenylindole) (Invitrogen) and images were captured either

using the TissueFax (TissueGnostics) fluorescence microscope or the Nikon A1 confocal laser microscope system.

Results

Specificity of TCR-like antibodies

The antibodies that recognize the HBV peptides $Core_{18-27}$ and $Env_{183-191}$ in complex with HLA-A2 MHC class I molecule were isolated by Konduru S.R. Sastry from mice separately immunized with the refolded pMHC complexes. A schematic representation of the production of the antibodies is shown in Figure 2.1. In summary, about 1500 and 2000 hybridomas were produced from mice immunized with the $Core_{18-27}$ HLA-A2 (Core18/A2) and $Env_{183-191}$ (Env183/A2) HLA-A2 complexes, respectively. The majority of these did not secret antibodies that bound to the peptide-HLA-A2 complexes, and of those that did, most were not peptide specific, as expected (Table 2.1B). However, several of the antibodies did exhibit peptide specificity, and from these, a single hybridoma clone was selected for each peptide-HLA-A2 complex, and the antibodies were purified by chromatography from culture supernatant. These two antibodies were used for the studies presented in this chapter.

Hybridoma screening during the generation of the antibodies showed that the antibodies had specificity for their cognate ligand, but to further examine specificity and possible cross-reactivity, the antibodies were tested with a panel of known HLA-A2 binding peptides including HBV peptides derived from the polymerase, X, envelope and core proteins, as well as other viruses and tumor associated antigens. Figure 2.2A

shows that both antibodies were highly specific for their respective peptide MHC and exhibit minimal if any cross-reactivity to the other HLA-A2 binding peptides.

Next, the antibodies were tested for cross-reactivity with other MHC class I molecules. Several EBV transformed B cell lines expressing the natural allelic variants of the HLA-A2 Class I MHC HLA-A*02:01, -A*02:02, -A*02:03 and -A*02:06, along with cells that express two other serotypes; -A*11:01 and -A*24:02 were pulsed with 1μM of peptides before staining with the respective antibody. The antibodies showed cross-reactivity with HLA-A*02:01 and -A*02:02 but minimal binding to the other MHC Class I complexes (Figure 2.2B). Although A*02:03 and A*02:06 share greater homology to A*02:01 and A*02:02 than A*11:01 and A*24:02 (Figures 2.2C,D), the mutations on the α2 domain helix in A*02:03 potentially affect the antibody recognition while the F9Y mutation in A*02:06, found on the beta sheet at the base of the peptide binding groove, could potentially affect the way the peptide is presented to the antibodies.

Binding avidity of TCR-like antibodies

To determine the binding affinity of the antibodies for their respective pMHC ligands, two separate experiments were done. First, T2 cells pulsed with 1μM of $Core_{18-17}$ or $Env_{183-191}$ peptides were stained with a titration of concentrations of the respective antibody as described in Materials and Methods and analyzed by flow cytometry. The mean fluorescent intensity of binding was plot against the concentrate of antibody used (Figure 2.3A). Binding kinetics were further analyzed by surface plasmon resonance (SPR). Soluble Core18/A2 and Env183/A2 pMHC complexes were refolded *in vitro* and immobilized to CM5 biosensor chips by amine coupling. Binding studies of the

antibodies to these immobilized pMHCs were then carried out with varying concentrations of antibodies. SPR measurements suggest that both antibodies had apparent K_D values between 20-50nM (Figure 2.3B), indicating that both antibodies had relatively high affinities for the pMHC ligands.

Fine specificities of TCR-like antibodies

TCRs and antibodies are structurally similar but recognize and bind their respective antigens in distinct manner. While TCR recognize their pMHC ligands in fixed diagonal orientations with respect to the MHC helices, studies to date suggest that antibodies recognize ligands with more variable docking orientations (23, 127, 169). Thus, with the availability of these two TCR-like antibodies, it is of interest to compare the possible footprints of these antibodies, over their respective pMHC complexes, with two TCRs with the same specificity. In addition, it would be useful to compare the peptide fine specificities of the antibodies and TCRs, to possibly understand aspects of the binding sites of these distinct molecules.

To begin to examine these issues, peptide variants of the two HBV peptides with single amino acid substitutions in every position in the amino acid sequences of the peptides were generated. The ability of the antibodies to recognize and bind these peptide variants in complex with the HLA-A2 class I molecule were tested using T2 cells pulsed with 1μM of each peptide. Figure 2.4A shows the Core18/A2 antibody did not tolerate amino acid substitutions are position 22 and 23 while the binding of the Env183/A2 antibody was affected by substitutions at positions 187 and 189. T2 cells pulsed with the alanine peptide variants were also used in degranulation assays with

two monoclonal T cell lines that express TCRs that recognize the Core18/A2 and Env183/A2 complexes respectively. Activation of these cell lines was measured by the CD107 expression. Figure 2.4B shows the effect of the amino acid substitutions on the activation of the monoclonal T cell lines expressing TCRs that recognize the Core18/A2 and Env183/A2 complexes respectively (Core18/A2 T cell activation data provided by Adam Gehring). Interestingly, similar to the antibody, activation of the Core18/A2 specific T cell clone was most affected by positions 22 and 23. However, the Env183/A2 specific T cell had a very different footprint or chemistry of binding compared to Env183/A2 antibody with positions 186 and 188 affecting binding most while positions 187 and 189 were less important. Furthermore, it can be observed that T cell activation was affected by substitutions at more positions than antibody binding, although this could also be attributed to the inherent sensitivities of the T cell activation and antibody binding assays,

TCR-like antibodies recognize endogenously processed and presented pMHC in HBV infected cells

Although the antibodies have so far been shown to be able to recognize and bind HBV peptide pulsed T2 cells, broader applications of these antibodies would be possible if they actually can be used to detect pMHC complexes produced by HBV-infected hepatocytes (i.e. peptides from endogenously processed viral proteins). To do so, I utilized an HLA-$A2^+$ human hepatoma cell line HepG2 and a derivative cell line known as HepG2-H1.3 (H1.3). H1.3 cells are HepG2 cells stably transfected with a plasmid containing 1.3-fold over-length HBV genome (186) and thus are able to express

all HBV proteins and to present on their surface HBV peptides-HLA-A2 complexes. Production of HBV proteins are stimulated by culturing in Williams media with supplements as described in Materials and Methods. Figure 2.5 shows that both antibodies are able to recognize and bind pMHC complexes formed endogenously in stimulated H1.3 cells. However, the quantity of specific pMHC complexes produced by the H1.3 cells is lower than peptide pulsed HepG2 cells, in part possibly due the observations that hepatocytes have inefficient antigen processing machinery (181).

Visualization of pMHC complexes in pulsed cells and virus producing cell lines by confocal microscopy

The ability of the antibodies to recognize both exogenously pulsed and endogenously processed pMHCs was demonstrated by flow cytometric analyses. Next, I determined whether the antibodies could be used to visualize these pMHCs by microscopy. Adherent HepG2 and HepG2 H1.3 cells were cultured over coverslips. HepG2 cells were pulsed or left un-pulsed while H1.3 cells were stimulated for production of HBV proteins. The coverslips were then fixed and stained with the respective antibodies. A tyramide signal amplification kit was used to visualize the antibody staining. Coverslips were mounted in reagent that contained DAPI for the visualization of nuclei. Figure 2.6A and Figure 2.7A show the visualization of the stained cells under a fluorescent microscope using the Core18/A2 antibody and Env183/A2 antibody respectively.

Pulsed and un-pulsed HepG2 cells were further analysed by confocal microscopy, using the Core18/A2 antibody and an antibody specific for the MHC class I

subunit β_2-microglobulin (β_2m). As shown in Figure 2.6B, staining of the Core18/A2 antibody co-localized with that of an antibody against β_2m, providing further evidence that the antibody was specifically binding the pMHC on the surface of the pulsed HepG2 cells.

Immunohistochemistry is often done on patient biopsies during diagnosis. To determine if the antibodies might be amenable to this application, cryostat sections of liver biopsy specimens from chronic hepatitis B patients were stained with the Env183/A2 antibody (data provided by collaborators from Barts and the London School of Medicine, UK) and analysed using the EnVision Plus mouse HRP polymer kit. Figure 2.7B shows that the antibody specifically stained naturally infected cells from patients that were antigen and HLA-A2$^+$ but not specimens from antigen negative or HLA-A2$^-$ patients.

Intracellular Toll-like receptors such as TLRs -3, 7, 8 and 9 are innate immune receptors that detect the presence of viruses by the recognizing viral DNA or RNA in the endosomal compartment and initiate anti-viral responses including the production of type I interferons (24, 96). However, the hepatitis B virus has evolved strategies to evade detection by TLRs (179, 218). Thus the option to stimulate Toll-like receptors signalling in infected cells by the directed delivery of TLR agonists provides a possible therapeutic measure to aid in the control of HBV replication in the infected cell. Antibodies are known to internalize by receptor-mediated endocytosis. To examine if the TCR-like antibodies were similarly internalized in HBV producing HepG2 cells after binding their pMHC targets, Alexa-488 fluorophores conjugated Env183/A2 antibodies were first prepared by Zenon labelling (Invitrogen). Target cells grown on cover slips

were first stained with the conjugated antibodies and a membrane dye as described in Materials and Methods and then incubated in culture media at 37°C. Coverslips were then washed and further stained with DiI red for the plasma membrane or LysoTracker red (Invitrogen) for lysosomes before mounting and analysis by confocal microscopy. Figure 2.7C shows that surface staining of the antibody co-localized with the membrane dye DiI red at the beginning of the incubation but diminished after 4hrs and appear to be internalized due to the loss of co-localization with DiI red staining. At 16hrs, cells were stained for lysosomes using LysoTracker red and in Figure 2.7D, the antibodies that had internalized are observed to be located in the lysosomes, evidenced by the co-localization of the Alexa-488 fluorophores and the LysoTracker red staining (internalization data provided by Konduru Sastry).

Quantifying pMHC complexes required for T cell activation on pulsed cells

The activation of $CD8^+$ T cells depends on the interaction of co-stimulatory molecules, the affinity of the TCR for the pMHC, and the density of the peptide-class I MHC complex (105). The number of pMHC required for the activation of T cells has been observed to vary from several thousands to as low as three per target cell, depending on the pMHC-TCR pair (105, 192). Here, I utilized the TCR-like antibodies to investigate the sensitivities of two T cell clones that are also specific for the Core18/A2 and Env183/A2 pMHCs (Figure 2.8). Quantification of the number of complexes on the surface of pulsed T2 cells was done by comparing the mean fluorescence intensity (MFI) of the same fluorophore conjugated-secondary antibody bound to TCR-like antibody associated on the surface of pulsed cells with those bound to standard beads

conjugated with known number of mouse IgG (Dako QIFI™ kit). T2 cells were first pulsed with peptide concentrations ranging from 1fM to 1μM and then stained with saturating concentrations of TCR-like antibodies as described in Material and Methods. These stained cells and the standard beads were then incubated with the same secondary antibodies and finally analysed by flow cytometry. A standard curve correlating the number of IgG on each population of bead provided in the kit with the MFI of the staining by the secondary antibody was determined as specified by manufacturer's instruction and the number of pMHC complexes on each population of pulsed T2 cells was determined from this curve. Similarly pulsed T2 cells were used in CD8 T cell degranulation assays to determine the levels of T cell activation. Pulsed T2 cells were co-incubated with the respective CD8 T cell clones with phycoerythrin conjugated anti-CD107 antibody for 5 hours at 37°C. After incubation, cells were collected and stained phycoerythrin-Cy7 conjugated anti-CD8 antibody, washed and analysed by flow cytometry. Figure 2.8 summarizes the results from these assays. While T cell activation was detected when peptide concentrations used for pulsing was as low as 10fM, TCR-like antibody binding was detectable only when T2 cells were pulsed with at least 10pM of peptides. Quantification using the standard beads showed that antigen presenting cells with 50 or less number of complexes were able to stimulate T cells (Figure 2.8B). Interestingly, for 50% of Env183/A2 specific T cell clones to be activated, a pMHC density of between 50-200 complexes per cell was required, but for the same level of activation of C18/A2 specific T cells, the number of complexes required is much greater at between 1000 to 6000 complexes, reflecting the difference

in sensitivity of the two different T cell clones (T cell degranulation data provided by Zack Ho and Adam Gehring).

Effect of interferons α and γ on antigen presentation

Interferons (IFN) play a major role in the control of Hepatitis B virus infection. Both type I and II IFN produced endogenously have been correlated with efficient control and clearance of HBV; in addition, IFN-α is now a standard therapeutic treatment for chronic infections. Studies have suggested that the anti-viral effect of interferons may be due to their inhibitory effect on viral gene expression (87, 200). This reduction in gene expression may result in the reduction of immune recognition due to the lower amount of viral antigens available for presentation. However, interferons are also known to be potent upregulators of class I MHC molecules expression (26, 195). Thus, it was of interest to study the effect of interferons on specific pMHC presentation on infected cells. Utilizing the Core18/A2 TCR-like antibody as the detection reagent, I analysed the levels of the Core18/A2 pMHC on the surface of the HepG2 H1.3 cells, HepG2 cells that are endogenously expressing HBV proteins, following treatment with IFN-α and IFN-γ (Figure 2.9). H1.3 cells were treated with three different dosages of IFN-α and-γ at 10U/mL, 100U/mL and 1000U/mL. Treated cells were harvested at 0hrs, 6hrs, 12hrs, 24hrs and 48hrs after treatment and stained with an anti-HLA-A2 antibody and the Core18/A2 antibody as described in Materials and Methods. Figure 2.9A shows that both IFN-α and-γ on HepG2 cells upregulated HLA-A2 molecules in a dose dependent manner, with effects lasting even after 48hours. Similarly, in Figure 2.9B, IFN-α and-γ upregulated HLA-A2 expression in H1.3 cells as soon as 6hrs and

continued to do so after 48hrs of treatment. Figure 2.8C shows the corresponding levels of Core18/A2 pMHC. The levels of Core18/A2 pMHC increased after 6hrs, correspondingly to the increase in HLA-A2 expression. However, unlike HLA-A2 expression, the levels of Core18/A2 pMHC peaked after 6hrs and plateaued and even showed slight decrease with further incubation. This suggests that the levels of core antigen available for presentation had becoming limiting, possibly due to a decrease in viral gene expression, as described by others (87, 200).

Discussion

The ability to study complexes of HBV peptides with MHC products has been limited resulting in a lack of information on how the quantitative and distributive features of HBV pMHC on infected cells affects immune responses. To address this, two monoclonal murine antibodies specific for the immunodominant epitopes $Core_{18-27}$ and $Env_{183-191}$ peptides, restricted by the class I MHC molecule HLA-A2 were isolated in the lab. Here I show that not only are these antibodies specific for their respective pMHC ligands, but they have high sensitivity and are able to recognize both exogenously and endogenously formed pMHC complexes. The latter two points are of particular significance because of low levels of HLA class I molecule expression and poor antigen-processing activity of hepatocytes (79, 181). I also show here that these antibodies can be used in various applications involving HBV-related studies.

As a diagnostic tool, the antibodies exhibited excellent specificity and sensitivity not only with pulsed cells and transduced cell lines; they were also able to recognize pMHC complexes on naturally HBV-infected hepatocytes and be used in tissue biopsy

staining (Figure 2.7B). In addition to this, the ability to visualize pMHC on the surface of cells allows us to not only understand the distribution of specific pMHCs at the tissue level, but also at the cellular level. These findings provide some additional understanding of viral pMHC recognition and T cell activation, for example in the context of the minimal requirements for immunological synapse formation. TCR clustering has been considered a pre-requisite for signalling and subsequent T cell activation (184), facilitating phosphorylation between the CD3 tails and associated protein tyrosine kinases for the initiation of intracellular kinase cascade (120). Studies have suggested that TCR clusters are pre-formed and exist even in resting cells while others proposed some degree of valency of interaction between the pMHC and the TCR had to be formed within close contact regions between the antigen presenting cell and the T cell in order for activation to occur (5, 209). Regardless of the model proposed, the pMHC presents a myriad of peptides on their surface MHC molecules and for the rare specific endogenous pMHCs to be able to promote TCR aggregation in order for activation to occur, there is a strong suggestion of a similar arrangement of clustered pMHC on the APC membrane. Furthermore, previous studies, along with my observations (Figure 2.8), had shown that very small numbers of pMHC complexes are required for T cell activation (105, 192). Here, I have demonstrated the applications of the antibodies both in the quantification of pMHCs and visualization in fluorescence-microscopy. These potentially allow further studies with infected hepatocytes that would definitely be able to provide us with a better insight into both the distribution of endogenously generated pMHC on naturally infected cells and its on T cell recognition and activation.

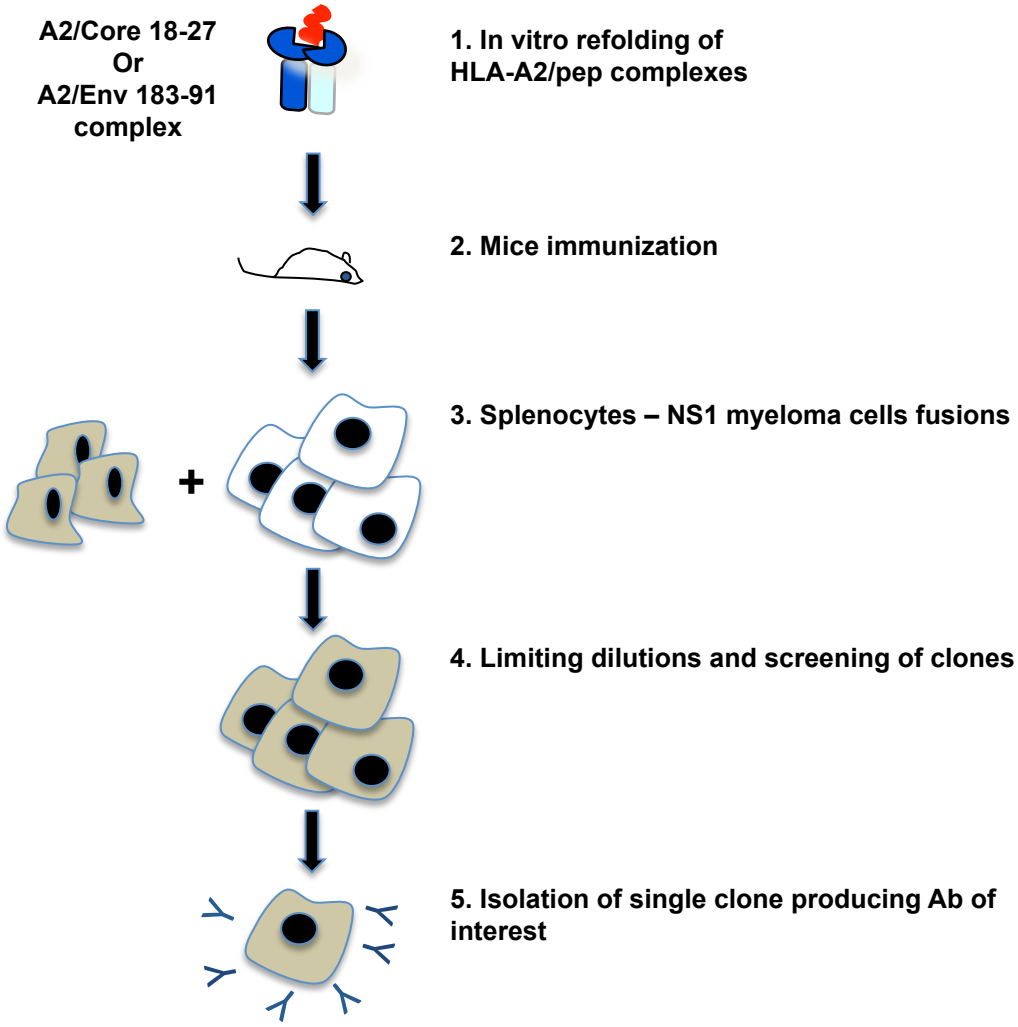
The antibodies also allow me to analyze the distribution and quantity of two immunodominant epitopes. These epitopes are recognized by the majority of $CD8^+$ T cells in infected patients (17, 124, 141) but such T cells are usually absent or in a tolerant state in chronic patients (161, 215). T cell clones that are specific for these immunodominant epitopes from infected patients have been previously isolated and I show, with the use of the antibodies, the high sensitivity avidity of these T cell clones for the pMHC complexes. More importantly, the T cell receptors of these T cell clones had been cloned and introduced into primary lymphocytes of chronic patients retrovirally (80). Not only were the TCR transduced T cells able to express the appropriate αβ TCR pair, they were able to recognize and lyse exogenously peptide pulsed hepatocytes and cell lines endogenously expressing HBV proteins, *in vitro and in vivo*. This study demonstrates the potential of adoptive transfer of TCR re-directed T cells for the treatment of HBV related hepatocellular carcinoma. In tandem with this notion, the antibodies specific for the same pMHCs can be used prior to administration of treatment in the diagnosis of patients. As shown in Figure 2.7B, biopsy analysis of specimens obtained from patients would facilitate informed decisions on the use of or the identification of the best HBV peptide-MHC target before proceeding with re-directed T cell therapy. The ability to quantify and compare the levels of HBV pMHC presented to the CD8 T cells, e.g. levels Env183/A2 pMHC and Core18/A2 allows for more personalized treatment with optimal efficacy. However notably TCR-like antibodies and TCRs may have different chemistries of binding for the same pMHC complex, e.g. the Env183/A2 antibody and the T cell clone and as shown by others (23, 99, 127, 134), thus the fine specificities of these antibodies have to be taken into consideration.

The use of these antibodies in the directed delivery of IFN-α in the form of fusion proteins had been demonstrated (102). However the evidence of internalization of the Env183/A2 specific antibody (Figure 2.7D) opens up the possibility of directed delivery of payloads such as Toll-like receptor agonists targeted at the endosomal TLRs -3, 7, 8 and 9. Intracellular TLRs function primarily to detect viruses with nucleic acids as ligands, however as the hepatitis B virus has evolved to evade detection by adopting a replication strategy whereby amplification of the viral genome occurs only within the capsids and the nucleus (179, 219). The ability to stimulate TLR signalling in infected cells would lead to the induction of genes involved in anti-viral responses including the production of type I interferons (24, 96). Type I interferons, IFN-α and IFN-β, are secreted by the virus-infected cells while type II, or IFN-γ is secreted by immune cells such as the $CD4^+$ Th_1 and $CD8^+$ T cells. Both groups of interferons play vital roles in anti-viral immunity; IFN-γ signalling itself affects more than 200 genes involved in various aspects of immune responses (26, 171). The suppressive effect of IFN-α on HBV replication has been studied extensively; with groups showing that one of the key effects is the downregulation viral protein expression (16, 87, 155, 200), leading to it being sanctioned for use as a therapeutic drug for chronic HBV. One function in common between the two interferons is their effects on class I antigen presentation. Both interferons upregulate the expression of MHC class I molecules while IFN-γ, in addition, also upregulates the expression of the transporter associated with antigen processing molecules (TAP1 and 2), tapasin, β-subunits and activator (PA28) of the proteasome; all are vital factors in antigen processing in the class I antigen presentation pathway (26). In concurrence with the former, I had demonstrated that both IFN-α and

IFN-γ are able to upregulate the expression of the HLA-A2 class I molecule in both HepG2 and HepG2 H1.3 cells that are expressing HBV. Guidotti et al had shown that IFN-α is able to downregulate HBV gene expression *in vivo*, while Tur-Kaspa *et al* presented evidence suggesting IFN-α reduced HBV gene transcription via the HBV enhancer (87, 200). Here, I show that in HepG2 cells expressing HBV proteins, treatment of both IFN-α and IFN-γ resulted in increased numbers of specific HBV Core18/A2 complexes on the surface of the cells. Hepatocytes express low levels of class I molecules, thus my observations would suggest that the poor expression of these molecules may be the limiting factor to the presentation of HBV specific pMHC on the surface of infected cells and with treatment of interferons, more such pMHC can be generated and presented, allowing infected cells to be more visible to T cells and facilitating the induction of anti-viral immune responses.

Figures

A



B

	Total	Negative	HLA-A2 Specific	pMHC specific
Core18-27	1452	1200	249	3
Env183-91	2000	1650	348	2

Figure 2.1. Generation of monoclonal antibody with TCR-like specificity for HBV Core18-27 and Env183-191 HLA-A2 peptide MHC complex. (A) Flow chart of the isolation of monoclonal antibody produced from a single hybridoma clone made from splenocytes of mice immunized with refolded pMHC. (B) Number of hybridomas screened during isolation and final number of pMHC specific cloned isolated. Data from Sastry Konduro.

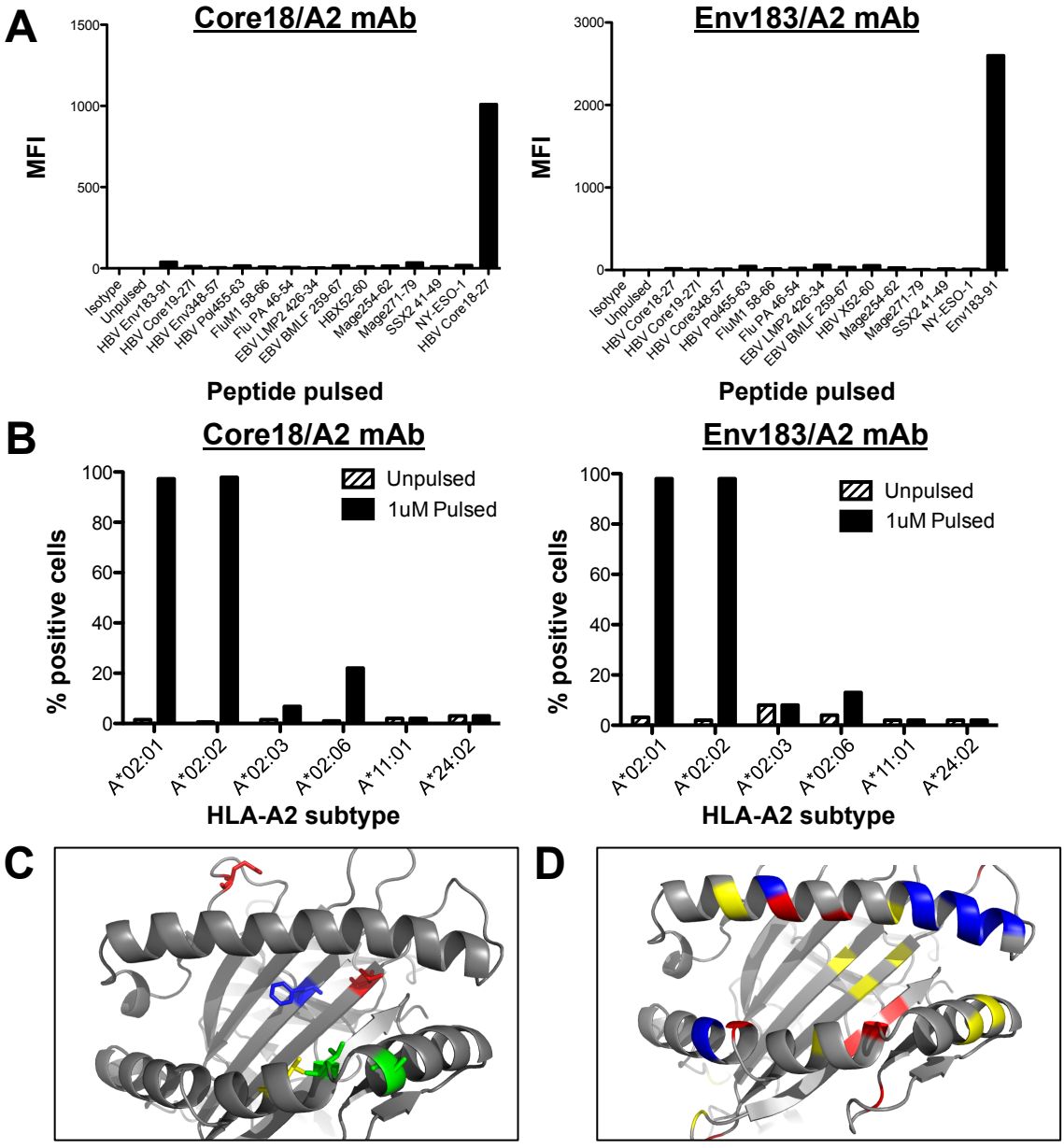


Figure 2.2. Specificity of TCR like antibodies that recognize 2 hepatitis B virus peptides restricted by the HLA-A2 MHC molecule. (A) Both Core18/ A2 antibody and Env183/A2 antibody showed specificity for their cognate pMHC ligand and no cross-reactivity for the other pMHC tested. (B) EBV immortalized B cell lines positive for different HLA-A2 subtypes and 2 other HLA-A allomorphs were pulsed with 1uM of either the $Core_{18\text{-}27}$ or $Env_{183\text{-}191}$ peptides and stained with the respective antibody. Both antibody recognize their cognate peptides presented by HLA-A*02:01 and HLA-A*02:02 subtypes but not the other MHC class I molecules. Amino acids positions that differ from HLA-A*02:01 (grey) are indicated for (C) A*02:02 (red and yellow), A*02:03 (green and yellow), A*02:06 (blue) and (D) HLA-A*11:01 (red and yellow) and A*24:02 (blue and yellow).

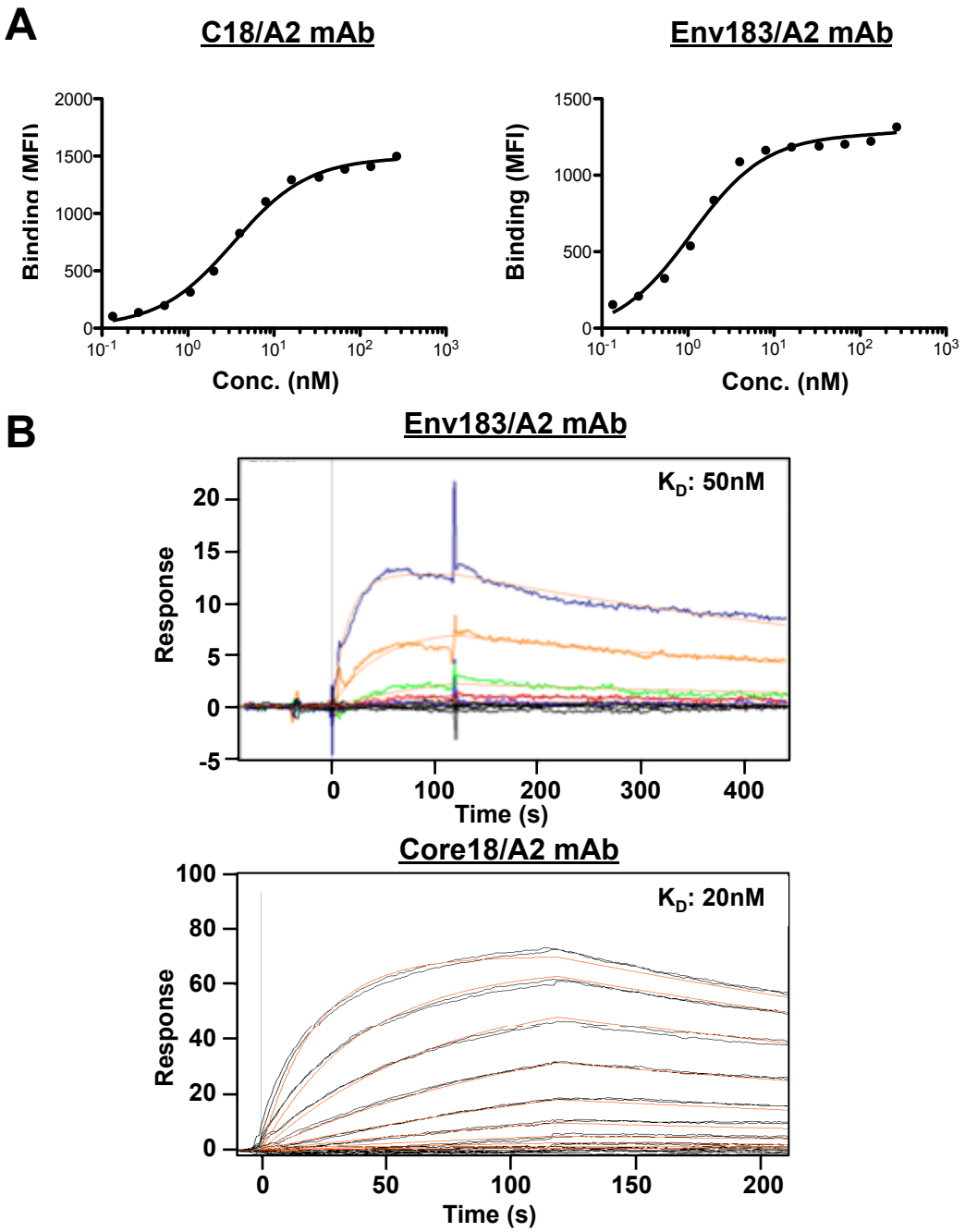


Figure 2.3. Measuring the dissociation constant (K_D) of the TCR like antibodies. (A) T2 cells were pulsed with 1μM of either the $Core_{18\text{-}27}$ or the $Env_{183\text{-}191}$ peptide and stained with a titration of the respective TCR like antibody. (B) Binding kinetics were further analyzed by surface plasmon resonance. Titration of antibodies were flowed over Core18/A2 or Env183/A2 pMHC immobilized on the surface of sensor chips and the rates of binding and dissociation were used in the calculation of the respective dissociation constant K_D.

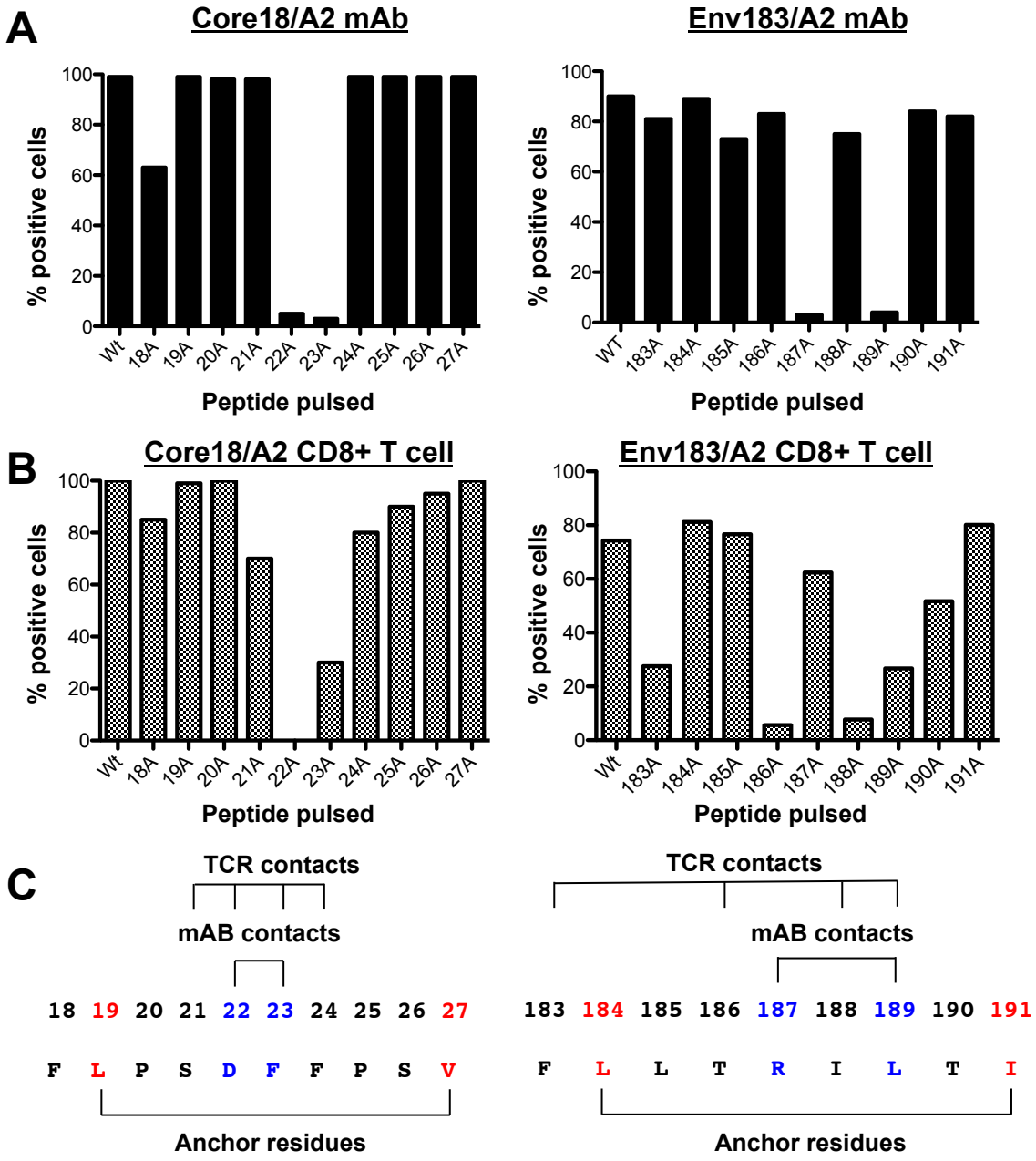


Figure 2.4. Comparison of fine specificity of TCR like antibodies and respective T cell receptors. Single position alanine substitutions were made at every position of the Core$_{18\text{-}27}$ and $_{Env183\text{-}191}$ peptides. (A) T2 cells pulsed with each peptide variant were stained with the respective TCR like antibody. Substitutions at positions 22 and 23 affected Core18/A2 antibody binding while positions 187 and 189 affected Env183/A2 antibody binding. (B) The same peptide variants were used in T cell activation assays with T cell clones specific for the same peptide epitopes. (C) Comparison of positions that significantly affected T cell activation or the respective TCR-like antibody binding.

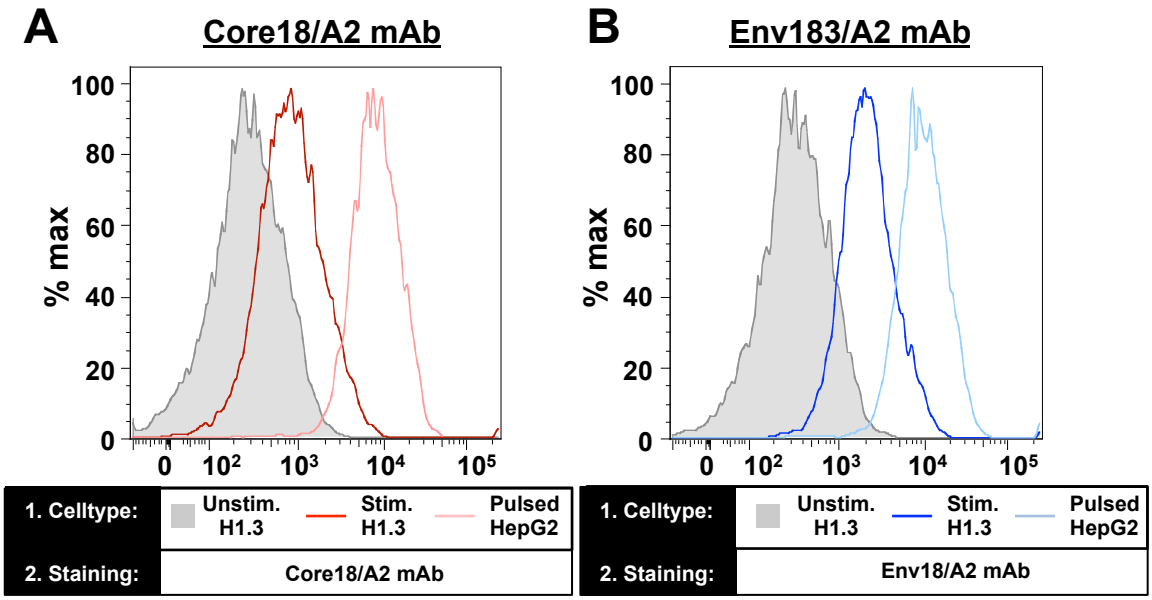


Figure 2.5. TCR like antibodies recognize and bind both exogenously pulsed and endogenously process pMHC complexes. HLA-A2^{+} HepG2 H1.3 cells that express HBV proteins and thus present HBV viral pMHCs on their cell surface were stained with either (A) Core18/A2 antibody or the (B) Env183/A2 antibody. HepG2 cells pulsed with exogenous the $Core_{18\text{-}27}$ and $Env_{183\text{-}191}$ peptides were also stained with the the respective antibodies showing that both antibodies are able to recognize and bind endogenous and exogenously formed pMHC complexes.

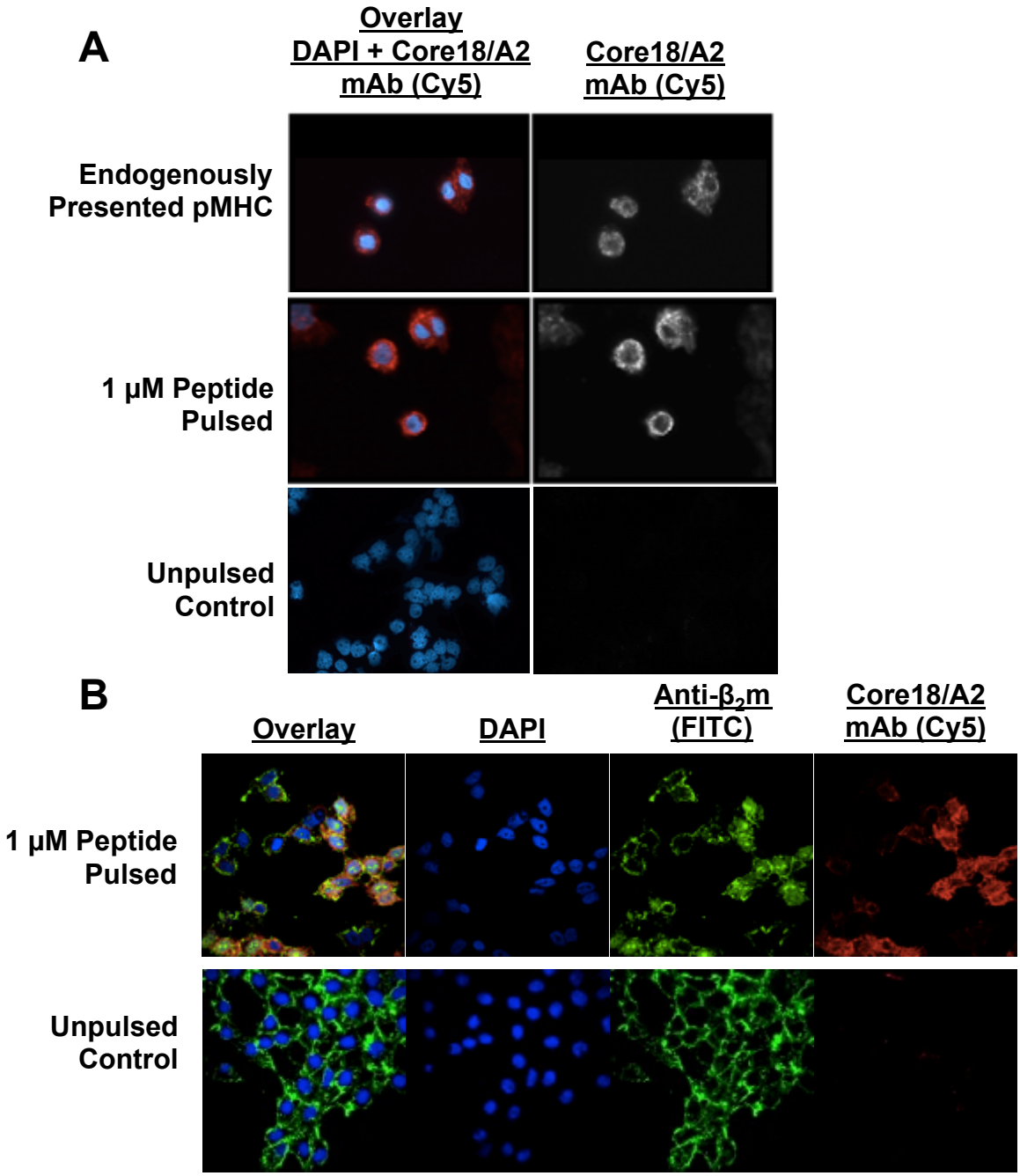


Figure 2.6. Visualization of endogenously processed and exogenously pulsed Core$_{18\text{-}27}$ pMHC on the surface of cells. (A) HepG2 H1.3 cells, unpulsed and 1μM Core$_{18\text{-}27}$ peptide pulsed HepG2 cells were stained with the Core18/A2 antibody and visualized by fluorescent microscopy. (B) Unpulsed and pulsed HepG2 cells were co-stained with the Core18/A2 antibody and an anti-β_2m antibody and visualized by confocal microscopy. Co-localization of anti-β_2m antibody and Core18/A2 antibody staining shows that the TCR like antibody was specific for the pMHC complexes.

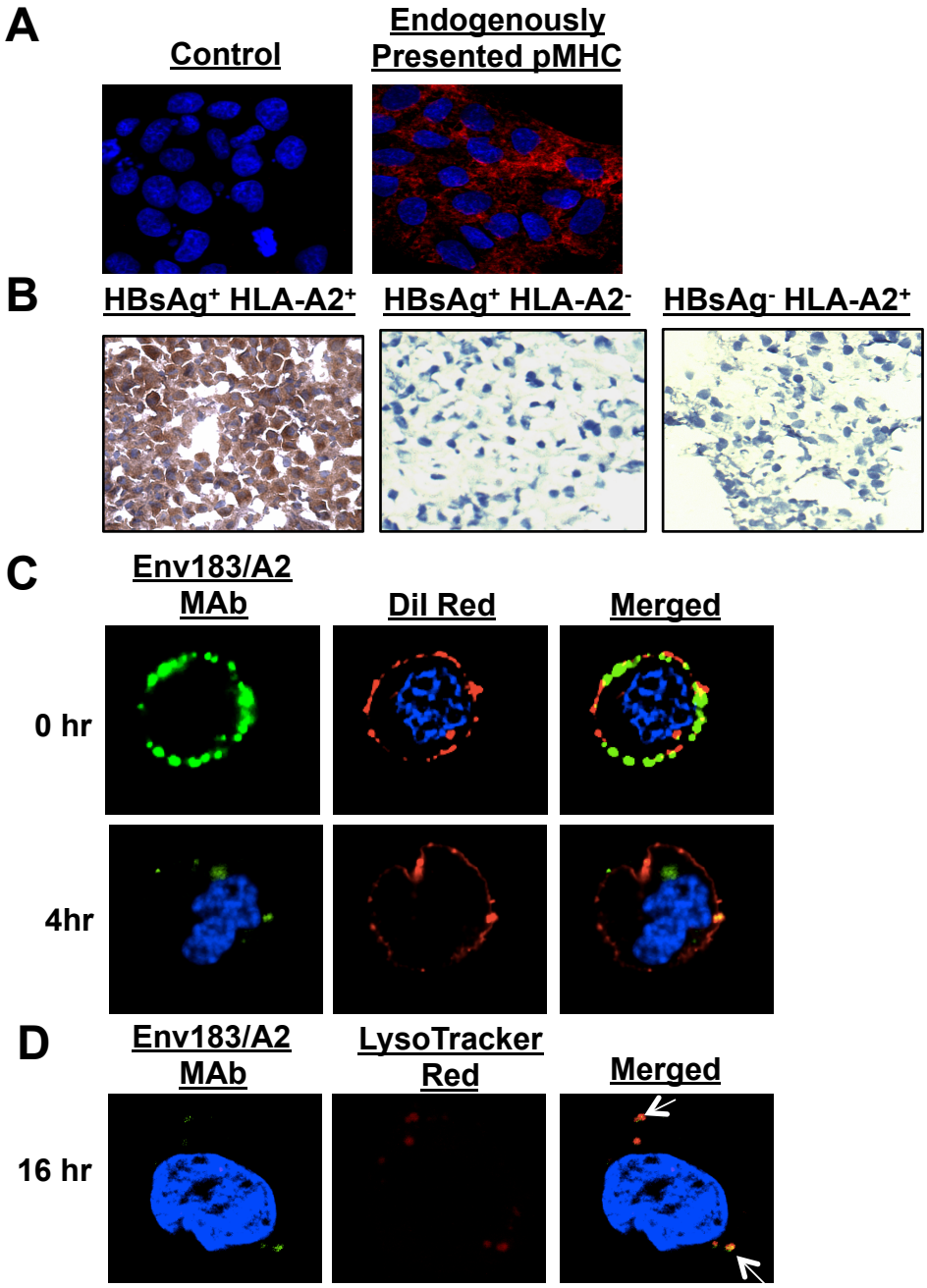


Figure 2.7. Visualization of $Env_{183-191}$ pMHC on the surface of infected cells and internalization of Env183/A2 antibody. (A) HepG2-117 cell lines that express HBV proteins were stained Env183/A2 antibody or and isotype control antibody and visualized by fluorescent microscopy. (B) Liver biopsy sections were stained with Env183/A2 antibody detected by immunohistochemistry. (C) Env183/A2 bound to the surface of HepG2-117 cells were observed to internalized after 4hr incubation at 37°C. (D) Fluorescently conjugated Env183/A2 co-localized with lysosome staining after 16hr incubation.

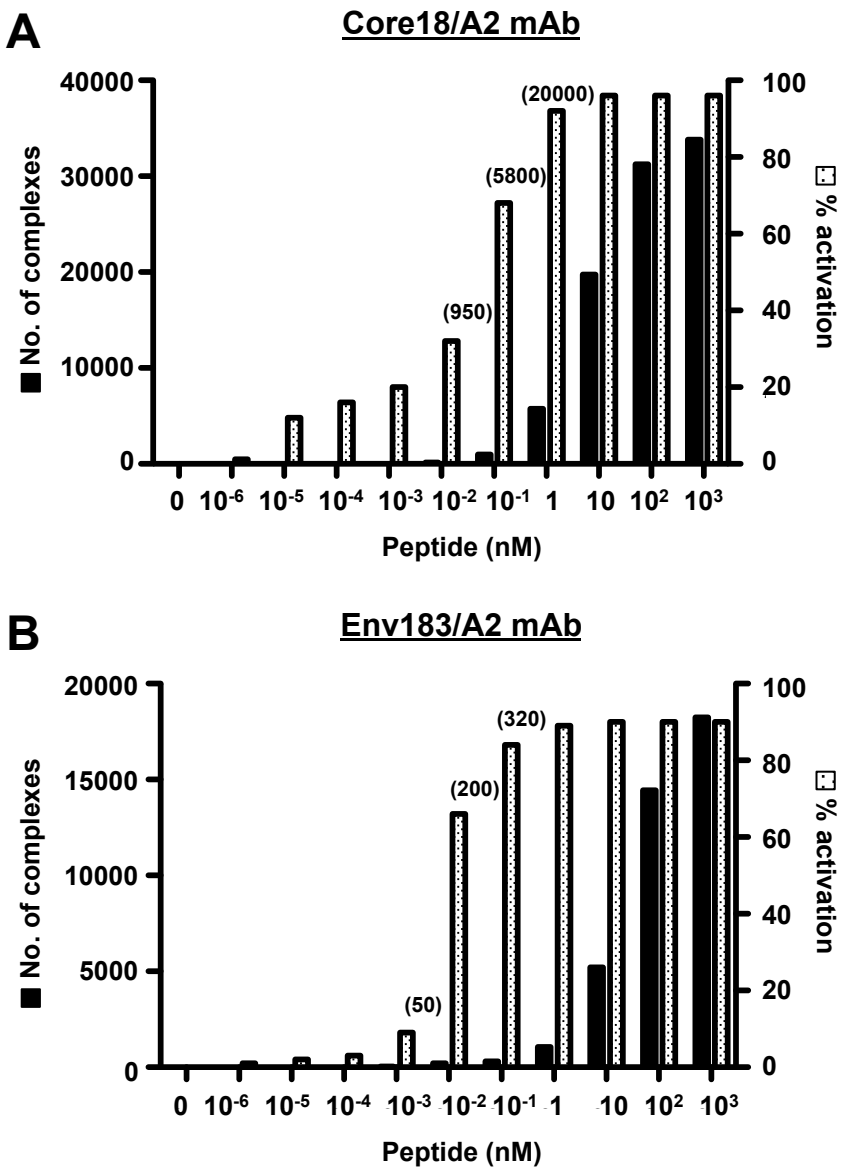


Figure 2.8. Quantifying pMHC complexes required for T cell activation on pulsed cells. T2 cells were pulsed with (A) $Core_{18-27}$ and (B) $Env_{183-191}$ peptide concentrations ranging from 1fM to 1μM and stained with the respective antibody or used for T cell degranulation assay with T cell clones specific for the respective pMHC. Number of specific HBV pMHC complexes presented on the surface of the T2 cells were further quantitated using the Dako QIFI™ kit and correlated with the corresponding level of T cell activation.

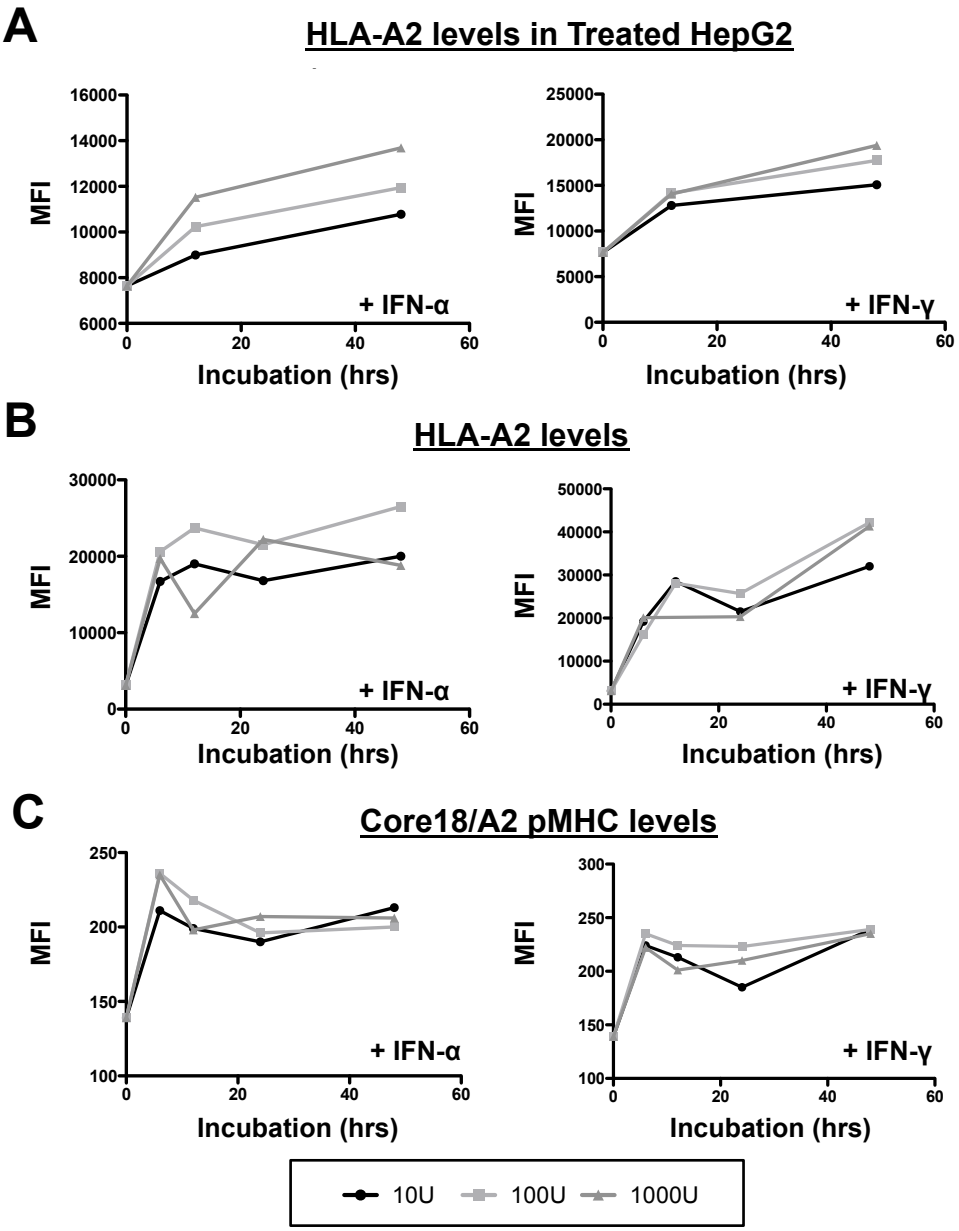


Figure 2.9. Effect of interferons alpha and gamma on the presentation of specific pMHCs. (A) HepG2 cells were treated with three concentrations of IFN-α or IFN-γ, and levels of HLA-A2 were probed at three time points 0hrs, 12 and 48 hours after treatment by staining with an anti-HLA-A2 antibody. (B) HepG2 H1.3 cells were similarly treated with three different concentrations of interferons and HLA-A2 levels were detected 0, 6, 12, 24 and 48 hours after incubation. (C) Levels of specific $Core_{18\text{-}27}$ HLA-A2 complexes were also probed at the same time points as (B) using the Core18/A2 antibody.

CHAPTER THREE

COMPARISON OF A SOLUBLE T CELL RECEPTOR AND A T CELL RECEPTOR-LIKE ANTIBODY SPECIFIC FOR HEPATITIS B/HLA-A2

Introduction

Antibodies and T cell receptors (TCRs) represent two distinct classes of immune molecules that the adaptive immune system in mammals has evolved to recognize foreign pathogens. Whereas antibodies can function as soluble molecules, TCRs are found only as membrane bound receptors (129). Furthermore, while antibodies are able to recognize antigens alone, often as linear or conformational epitopes of a polypeptide, TCR recognition invariably requires the antigenic peptide to be presented by a major histocompatibility complex (MHC) product on the cell surface (55). For example, peptide-MHC (pMHC) complexes are formed in virus-infected cells when processed viral proteins are loaded onto class I MHCs and delivered to the cell surface (154). This enables infected cells to be recognized, typically by a $CD8^+$ T cell, resulting, ideally, in activation of the T cell, destruction of the target cell, and clearance of the virus (182). In hepatitis B virus (HBV) infection, which still poses a global health problem despite the availability of an effective prophylactic vaccine, $CD8^+$ cytotoxic T lymphocytes (CTLs) play a crucial role in the antiviral response (21). Effective control of HBV relies on robust CTL responses, and $CD8^+$ T cell dysfunction is associated with chronic infection, which in turn carries the risk of complications such as hepatocellular carcinoma.

The ability to activate CTLs and the magnitude of CTL responses correlates with the levels and density of pMHC presented by HBV-infected cells (79). This has raised

an interest to both visualize the presented antigens on the surface of infected hepatocytes, and to generate molecular entities that enable the targeted delivery of pharmaceutical or immunomodulatory compounds (102, 172). A promising approach uses monoclonal antibodies (mAbs) with TCR-like specificities for distinct pMHC products, which attain affinities higher than normal TCRs (139, 149, 172). For such novel TCR-like mAbs, however, it remains important to individually assess whether they exhibit the same specificity as the cognate TCRs (76, 130). Structural studies already demonstrated that the docking position can differ from the conventional TCR, exhibiting interactions predominantly with the MHC, thus affecting peptide specificity (99). In addition, TCR-like mAbs are often raised in non-human species (*e.g.* mouse, rat or rabbit) and require subsequent transformation into human chimeras; a process known as “antibody humanization”, to be of therapeutic value. Because such mAbs have not been selected *in vivo* in the target species, cross-reactivity with self-antigens can also formally not be ruled out.

An alternative strategy employs multivalent soluble TCRs to target antigens presented on the cell surface (9, 115), analogously to probing clonotypically expressed TCRs with oligomeric pMHC molecules, which are routinely used for the identification and characterization of virus-specific CTLs (6, 54). For successful implementation, however, several technical limitations need to be addressed. First, the expression and production of soluble TCRs is often problematic (169). Second, the affinities of TCRs are normally in the micromolar range, making detection of pMHC on cell surfaces with a soluble TCR probe difficult (132). Third, while the TCR is expressed on T cells at a uniform, relatively high density (about 50,000 molecules per cell; (189)) the number of

naturally processed antigenic pMHC complexes per cell is thought to be low, in the range of 10 to 1000 (226). To overcome these challenges, soluble TCRs have been recombinantly produced in various systems, such as insect cells (77) and *E. coli* (30, 56, 75, 175). The low affinities of the monomeric TCRs have been enhanced both by oligomerization (9, 115, 143, 190, 230), and by directed evolution in either yeast or phage display systems (95, 118).

In an effort to develop novel protein constructs for diagnostic and therapeutic application in the context of HBV, we generated a soluble form of an αβ T cell receptor isolated from an HLA-A*02:01 restricted $CD8^+$ T cell clone specific for the immune-dominant $Env_{183-191}$ epitope (Env183/A2) of the HBV envelope protein. We show that the soluble TCR is both functional and highly peptide-specific using a multivalent, bead-based assay that we developed as a novel platform for probing TCR:pMHC interactions. Using this assay, we compared the $Env_{183-191}$ peptide fine-specificity of both the TCR and a monoclonal antibody previously isolated to recognize the same $Env_{183-191}$/HLA-A*02:01 (172). Finally, we established that the surface density of HLA complexes is a critical factor when TCR multimers and TCR-like mAbs are employed to detect MHC-presented peptide antigens.

Materials and Methods

Cloning of TCR α and β *chains*

The Vα34 and Vβ28 Env183/A2 specific heterodimeric TCR was previously isolated from a T cell clone (80). Each chain was truncated, the α chain after Ser202 and the β chain after Asp243, and cloned into pET28a expression vectors separately by

PCR using 5' primers containing an NcoI restriction enzyme site that also contains the ATG start codon and 3' primers that contains the stop codons and an XhoI restriction enzyme site. A disulfide bond was introduced by mutating residues Thr48 of the α-chain constant domain and Ser57 of the β-chain constant domain into Cys residues (as described previously (30)). In addition, an unpaired Cys75 of the β-chain constant domain was mutated to a serine to facilitate the specific pairing of the introduced Cys residues. A biotinylation motif was added to the C-terminal end of the β-chain to facilitate tetramerization with streptavidin

Expression of TCR chains

The TCR chains were separately expressed in Rosetta™ Competent *Escherichia coli* cells (Novagen). Inclusion bodies were harvested from cells lysed by sonication and washed using washing buffer (50mM Tris, 100mM NaCl, 0.5% Triton X-100, 1mM EDTA, 0.1% sodium azide). 10mg/L of biotin was added during induction to the β chain expression cultures to facilitate *in vivo* biotinylation. Inclusion pellets were then dissolved completely in urea solution (8M urea, 25mM MES, 10mM EDTA, 0.1mM DTT, pH 6.0). Solubilized inclusion bodies were quantitated by Bradford assay (Biorad, Richmond, California) and stored at -80°C.

Refolding and purification of refolded TCRs

The soluble TCR was refolded by rapidly diluting aliquots of 10mg of each chain in 1 litre of refold buffer (100mM Tris pH 8.0, 400mM L-Arg HCl, 2mM EDTA, 5mM reduced glutathione, 50mM oxidized glutathione) supplemented with a tablet of

cOmplete ULTRA protease inhibitor cocktail (Roche Applied Science). Two further injections of each inclusion bodies 8hrs apart yielded a final concentration of 60mg/mL total protein. The refolded protein was concentrated using a Vivaflow 50 crossflow cassette (Sartorius Biotech) and dialyzed twice into 2.5 litres of 20mM Tris pH 8.0 overnight. Enzymatic biotinylation was carried out according to manufacturer's instructions using BirA biotin ligase (Avidity). Purification of biotinylated TCRs was carried out by size exclusion chromatography (HiPrep 16/60 Sephacryl S200) followed by anion exchange chromatography (MonoQ 5/50 GL) using an AktaFPLC system. Fractions containing refolded heterodimeric TCRs were identified by SDS-PAGE. Biotinylation of purified refolded TCR was probed by gel shift assay whereby refolded TCR prepared in non-reducing SDS-PAGE loading buffer were incubated with excess streptavidin at room temperature (RT) for 15mins before SDS-PAGE analysis.

Multimerization of refolded TCRs

Soluble TCRs were tetramerized by the addition of allophycocyanin (APC) conjugated-streptavidin (Invitrogen) to the monomers to a final volume of 1:4 respectively. The total amount of streptavidin-APC required was divided into 5 aliquots and added to TCR monomers in 30mins interval. TCR tetramers were then stored at 4°C and used within a month.

TCR-like antibody

A mouse monoclonal antibody specific for the HLA-A*02:01-restricted $Env_{183-191}$ epitope was previously generated (172). Antibodies were purified from the supernatant

of the hybridoma culture using a protein G-agarose column, concentrated and quantified by Bradford Assay (Biorad, Richmond, California).

Peptide-MHC conjugated beads assay

Recombinant HLA-A*02:01 MHC was expressed, refolded with the conditional ligand GILGFVF-J-L, biotinylated and purified as previously described (167, 199). UV-mediated peptide exchange of these cages pMHC with the required peptides was performed as reported (11, 44, 167, 199). Fluorescent streptavidin-coated yellow particles kits (Spherotech SVFA-2552-6K and SVFB-2552-6K), and non-fluorescent streptavidin-coated particles (Spherotech SVP-30-5) were used to capture the pMHC. Beads were pre-incubated with 200µL of blocking buffer (2% BSA in PBS) for 30mins with shaking followed by incubation in excess of pMHC for 1hr with shaking. All incubations were carried out at RT. For the titration of pMHC loaded onto the beads, 4 fold dilutions of the pMHC were done in blocking buffer before conjugation. Conjugated beads were washed with blocking buffer and subsequently stained with TCR tetramers of TCR-like antibodies. Staining of the conjugated beads was done at RT with shaking for 30mins before washing with blocking buffer and fixed with 1% paraformaldehyde in PBS before flow cytometry analysis. Secondary staining of the TCR-like antibody with an APC-conjugated goat anti-mouse antibody (Invitrogen) was done at RT with shaking for 20mins. Acquisition was done on an LSR-II or FACSCanto flow cytometer (Becton Dickinson) and data analysis was done off-line with FlowJo software (Tree Star) and GraphPad Prism software (GraphPad).

Cell culture and staining

T2 cells (ATCC CRL 1992) and EBO-PreS2 cells were cultured in RPMI 1640 medium while HepG2.2.15 cells were cultured in Dulbecco's modified Eagle's medium, both supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, 0.5 mM sodium pyruvate, minimal essential medium (MEM), nonessential amino acids, Glutamax, 5μg/mL Plasmocin (InvivoGen), 100 U/mL penicillin, and 100μg/mL streptomycin. Cells were treated with 100U/mL IFN-γ (R&D systems) for 24hrs before washing and pulsing. Cells were pulsed with 1μM or 10μM final concentration of peptides at RT before staining. Washing and staining were done in flow cytometry buffer (1% BSA, 0.01% sodium azide, in PBS). Cells were stained with 1ug/mL of Env183/A2 antibody or TCR tetramer unless otherwise stated at RT for 30mins with shaking. Secondary detection of TCR-like antibody was done with an APC conjugated goat anti-mouse antibody (Invitrogen).

Surface plasmon resonance

Binding studies were carried out using the BIAcore™ 3000 (BIAcore AB, GE Healthcare). HLA-A*02:01 monomers presenting either the $Env_{183-191}$ or $Core_{18-27}$ (control) peptide were immobilized onto CM5 sensor chips by standard amine coupling procedure. Briefly, the surface was activated for 5 min with 1:1 mixture of 0.2 M N-ethyl-N'-[3-(diethylamino)propyl]carbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS). The HLA complex, pre-diluted into 10 mM sodium acetate at pH 6.0, was then injected across the activated surface at 10 μL/min. The surface was finally blocked with by injection of 1 M ethanolamine at pH 8.5 for 7 min. Kinetic measurements were

performed in running buffer (50mM Tris, 150mM NaCl, pH7.0) at 30 μL/min at 25°C. TCR-tetramers (in 3-fold serial dilutions; 0.12, 0.37, 1.1, 3.3, 10 μM) were flowed over the immobilised HLA for 2 min and then allowed to dissociate for 30 min. The surface was regenerated with a 0.5 min pulse injection of 1:1000 phosphoric acid and stabilized with buffer for another 2 min before the next cycle. Binding responses were corrected against a control flow cell and a buffer cycle. Corrected responses were fit to a simple 1:1 bimolecular interaction model.

Structural modeling

The $Env_{183-191}$ peptide was modeled into the Tax-HLA-A*02:01 (PDB-ID:1DUZ) using the Rosetta Backrub program (http://kortemmelab.ucsf.edu) (183). Figures were made using Pymol (DeLano Scientific Research LLC).

Results

*Producing soluble $Env_{183-191}$ peptide/HLA-A*02:01-specific TCR tetramers*

The genes encoding the extracellular regions of the α and β chains of the Env183/A2 TCR (80), with an additional cysteine introduced in each C region (30), were cloned into pET28a vectors and the proteins were expressed in *E. coli* as inclusion bodies. The soluble α and β chains have predicted molecular weights of ~23kDa and ~28kDa, respectively. Equal amounts of inclusion bodies were combined under high dilution, and the concentrated solution was subjected to size exclusion chromatography (Fig. 3.1A). A peak corresponding to the expected size of the heterodimeric TCR (~51kDa) was subjected to anion exchange chromatography (Fig. 3.1B). Fractions

corresponding to observed peaks P1 and P2 were pooled, concentrated and analyzed by SDS-PAGE under reducing and non-reducing conditions (Fig. 3.2A). P1 appeared to contain predominantly β chain presumably representing soluble non-disufide linked β chain refolded monomers. The increased electrophoretic mobility under non-reducing SDS-PAGE of this fraction, compared to the inclusion body preparation, we attribute to intramolecular disulfide bonding. In contrast, P2 had the anticipated equal stoichiometry of α and β chain, corresponding to the disulfide-linked αβ heterodimer. All subsequent studies were performed with this complex. To generate multivalent forms of the TCR, a BirA biotin tag was included at the C terminus of the β chain. To assess if the purified protein (P2) had been biotinylated during recombinant expression, gel shift analysis was performed by incubation with strepavidin (Fig. 3.2B). This showed that the majority of the product “gel-shifted” which is indicative of biotinylation. Fluorescent TCR tetramers were produced by incubation with streptavidin-allophycocyanin (SA-APC) in a 4 to 1 stoichiometry, and to determine if the soluble TCR could bind its cognate ligand, surface plasmon resonance (SPR) analysis of TCR monomer and tetramers was performed by flowing them over immobilized Env183/A2 complexes. While monomer binding was below the detection limit compared to the control, specific binding of the TCR tetramers for the immobilized Env183/A2 complexes was detected (Fig. 3.2C).

A bead-based assay to probe pMHC binding to soluble TCR tetramers.

We explored an additional system to characterize the binding of soluble TCR and used streptavidin-coated beads conjugated with biotinylated soluble pMHC molecules (Fig. 3.3A) to provide a homogenous target with a high ligand density (44). Refolded,

caged HLA-A*02:01 was UV irradiated and peptide-exchanged with either $Env_{183-191}$ or $Core_{18-27}$ (control) peptide (11, 73, 83, 85, 199), immobilized on the beads and stained with either TCR monomers, followed by an APC-conjugated secondary detection Ab, or tetramers (Fig. 3.3B, C). In agreement with the SPR data, monomer binding was below the detection limit, but TCR tetramer staining of the Env183/A2-coupled beads was increased by two orders of magnitude (Fig. 3.3C) compared to beads coated with HLA-A*02:01 presenting the $Core_{18-27}$ peptide. This demonstrated that the bead assay provides a sensitive system for examining the specificity of soluble TCRs.

Consequently, different epitopes derived from the hepatitis B virus envelope, core and polymerase proteins, including two natural variants of the $Env_{183-191}$ epitope (Fig. 3.4A) were peptide-exchanged into HLA-A*02:01 complexes and conjugated to the streptavidin-coated beads. The levels of beta-2-microglobulin (β2m), indicative of MHC stability, showed that the number of pMHC molecules present was approximately equal for all pMHC products (Fig 3.4B). The TCR tetramer bound specifically both $Env_{183-191}$ peptide variants derived from two viral genotypes but not to the other HBV epitopes restricted by HLA*02:01 (Fig. 3.4C). The $Env_{183-191}$ epitope variants are found in either genotype A/C/D or genotype B viral strains and the TCR thus provides pan-specific recognition. This promiscuity is consistent with activation data of transduced T cells expressing the same α and β TCR genes (172).

TCR tetramers and the analogous TCR-like mAb have distinct fine-specificities.

It has been reported that a TCR-like monoclonal antibody (mAb) that recognizes the composite surface of HLA-A*02:01 presenting the $Env_{183-191}$ epitope (Env183/A2

mAb) was capable of binding only genotype A/C/D variants of the peptide epitope and not the genotype B variant (172). The difference between these two peptides lies in a single conserved amino acid at position 187 (Lys for genotype B and Arg for genotype A/C/D, Fig. 3.4A). Modeling studies revealed that the arginine side chain points away from HLA-A*02:01, and is solvent exposed, making it available for TCR interaction (Fig. 3.5A). To experimentally determine the fine-specificity, individual alanine substitutions at every position of the $Env_{183-191}$ peptide were generated and loaded into caged HLA-A*02:01 (Fig. 3.5B), which were then conjugated to beads and probed with either the TCR tetramers or Env183/A2 mAb. The levels of β2m were probed to ensure that the number of pMHC molecules on the surface was approximately equal for all pMHC products. Alanine substitution at position 187 had the largest impact on Env183/A2 mAb binding among all the alanine variants (Fig. 3.5C). While the TCR tetramer was able to tolerate the conservative amino acid variation between lysine and arginine (Fig. 3.4C), alanine substitutions had substantially reduced TCR tetramer binding (Fig. 3.5D). TCR tetramer binding appeared to be affected by alanine substitutions at all positions except the C-terminus. This observation agrees with T-cell activation data with transduced T cells expressing the same αβ TCR (172). The differences in binding to the presented peptide variants imply that although the two molecules bind the same pMHC ligand, they are likely to have different pMHC docking positions and/or different chemistries of interaction.

Detection limit of the bead-based assay

To determine the sensitivity of our assay, we titrated the soluble TCR tetramer, the mAb, and the amount of Env183/A2 complexes on beads. We multiplexed the system by employing a series of streptavidin-coated beads with varying fluorescent intensities. To examine the optimal concentration of TCR or mAb required for binding, the collection of beads were individually coated with saturating amounts of the Env183/A2 complexes. A titration of TCR tetramer demonstrated that TCR tetramers were able to bind their cognate pMHC at concentrations as low as 10 ng/mL (0.04nM) (Fig. 3.6A). The same collection of Env183/A2 strepavidin-coated beads was stained with the Env183/A2 mAb (Fig. 3.6B). Comparison of these titrations showed that at the same protein concentration both TCR tetramer and Env183/A2 mAb had similar avidity for the Env183/A2 products (Fig. 3.6C).

Subsequently, the influence of pMHC density on binding was examined with a fixed concentration of soluble TCR tetramer or Env183/A2 mAb. The bead populations were loaded with varying amounts of Env183/A2, providing an array of beads presenting pMHC at different densities. Staining with anti-β2m verified the decreasing amounts of pMHC on the surface (Fig. 3.7C), and allowed us to quantify the number of pMHC per bead (Table 3.1). This revealed that, at saturation, the beads displayed approximately 80,000 accessible pMHC. Staining these beads with the TCR tetramer (Fig. 3.7A) revealed that high densities of pMHC were required for adequate detection, with at least more than 3000 pMHCs on their surface (Fig. 3.7D). In contrast, staining with Env183/A2 mAb could be achieved at 10-fold lower pMHC density (Fig. 3.7B,D). This increased sensitivity of the mAb we ascribe to a higher intrinsic binding affinity.

*TCR tetramers bind cells only with high density of $Env_{183-191}$/HLA-A*02:01 complexes*

Given that the tetramer of the Env183/A2-specific TCR could bind immobilized pMHC, we next determined if it stained T2 cells (HLA-A*02:01$^+$, TAP deficient) pulsed with $Env_{183-191}$ peptide. As IFN-γ treatment is known to upregulate the number of MHC molecules on the cell surface (26), experiments were also performed with T2 cells incubated with IFN-γ followed by pulsing with peptide. Monomeric TCRs did not stain peptide-pulsed T2 cells (Figure 3.8A) whereas TCR tetramer yielded a moderate but distinct increase in fluorescence specific for the Env183/A2 complex at the highest peptide concentration tested (Fig. 3.8B). IFN-γ treated cells displayed an increase in staining with the TCR tetramer at both 1 and 10 μM $Env_{183-191}$ peptide (Fig. 3.8B). In contrast, the Env183/A2 mAb yielded a significantly greater shift in fluorescence, which was further increased upon IFN-γ treatment (Fig. 3.8C). The reduced staining by the TCR tetramer thus suggests that only a fraction of the Env183/A2 complexes on the T2 cells are capable of retaining bound TCR tetramer after washing, reflecting the low affinity of the TCR (189).

Furthermore, we compared the ability of the TCR tetramer and the Env183/A2 mAb to recognize Env183/A2 complexes produced endogenously by HBV-infected cells. We utilized an EBV-immortalized B cell line (EBO-PreS1) transfected with the open-reading frame for the PreS1 fragment of the HBV genome (88), which expresses only the envelope proteins together with the $Env_{183-191}$ epitope. In addition, HepG2.2.15 cells were used, that derive from a stably transfected HLA-A*02:01$^+$ hepatoma cell line bearing the complete HBV genome, expressing all viral RNAs and proteins (180). Both cell lines were treated with IFN-γ before staining. As the EBO-PreS1 and HepG2.2.15

cell lines are both TAP^+, they present self-peptides in addition to HBV epitopes and the steady state number of Env183/A2 ligands for the Env183/A2 antibody and the TCR tetramer should be considerably lower than peptide-pulsed cells (Fig. 3.9). Therefore, at best, minimal staining was observed with TCR tetramers, yet the Env183/A2 mAb stained both cell lines.

The beads with immobilized Env183/A2 complexes at various densities (Fig. 3.7D) along with the pulsed T2 cells results suggest that although the TCR tetramers bound specifically, the density of the HLA complexes critically influences their binding. Estimates of the density of Env183/A2 on the beads, with a surface area of ~25μm^2, are ~3200/μm^2 at their highest concentration. Assuming that an APC expresses 5-10 x 10^4 class I MHCs on average (227) which can be pulsed to saturation with $Env_{183-191}$ peptide, a density of 800/μm^2 can be achieved; which is sufficient only for detection by TCR-like mAb.

Discussion

T cell activation is preceded by several molecular interactions; the binding of the peptide to an MHC product, and the binding of a TCR to this pMHC complex. In the case of $CD8^+$ T cells, activation is also critically dependent on the binding of the CD8 co-receptor to the non-polymorphic regions of the class I MHC product. There has been considerable interest in using soluble extracellular domains of TCRs to probe T cell specificity and pMHC affinity independently from the CD8 binding synergism (43, 50, 71). Furthermore, soluble TCRs could in principle serve as reagents to probe surface expression of specific pMHC combinations on cells; particularly in cases where T cell activity assays are challenging (*e.g.* in immunohistological studies). In this study, the

binding and specificity of a hepatitis B virus-specific TCR was examined, and compared to a mAb recognizing the same $Env_{183-191}$/HLA-A*02:01 antigen.

We developed a system to sensitively discriminate the low affinity interactions between the pMHC and the targeting molecules. The assay employs streptavidin-coated beads to immobilize pMHC molecules at high density, similar to what can be achieved with an SPR chip-based approach. When probed with multivalent TCRs or TCR-like mAbs, the bead populations allow rapid determination of peptide fine-specificity by flow cytometry analysis. The bead-assay proved superior to using peptide-pulsed APCs, which was attributed to the high surface density of the antigen (*vide infra*). Furthermore, the polymer beads gave good control over the pMHC surface distribution, which was easily quantified, and allowed analysis of the specific interaction in the absence of adhesion molecules, co-receptors and pMHC products presenting alternative peptides, all of which are abundantly present on APCs.

The bead-based assay revealed that the HBV-specific TCR was properly assembled to recognize its cognate Env183/A2 antigen. Although the enhanced affinity of the TCR-like mAb holds promise for its application as sensitive probe or as targeting therapeutic, it differed from the corresponding TCR in its fine-specificity for the Env183/A2 antigen, most notably at the central 187 residue; a position where viral sequence variation is known to occur. The molecular model that we generated for the $Env_{183-191}$/HLA-A*02:01 complex prominently featured a protruding basic amino acid residue (*i.e.* Arg_{187} for HBV genotypes A/C/D, or Lys_{187} for HBV genotype B) from the binding pocket, and highlighted it as a important candidate residue for interaction with the TCR or the mAb. As the naturally occurring R187K mutation between HBV

genotypes A/C/D and genotype B already eliminated binding by the Env183/A2 mAb, the R187A substitution was equally disruptive to binding, as would be predicted (172). The TCR tetramers, on the other hand, bound both the R187 and K187 epitope variants, although the more profound R187A mutation did interfere with its specific recognition.

The half-life of TCR:pMHC interactions ranges in seconds, whereas oligomerization can extend that by several hundred-fold, thus enabling TCR tetramers to stably bind pMHCs long enough to employ them experimentally as surface staining reagents (115). The avidity gained through tetramerization of our Env183/A2-specific allowed the TCR tetramers to bind beads as well as the Env183/A2 mAb when saturated with the correct pMHC. The sensitivity of staining was clearly dependent on the degree of packing of pMHC on the beads, presumably because at higher density the TCR tetramer can bind clusters of pMHC simultaneously. In our hands, the TCR tetramer showed appreciable staining when loading of the individual beads, with a diameter of ~2.7μm, reached above 3,500 pMHC molecules. For a typical hepatocyte, with a diameter of ~20-40μm (58), the number of surface pMHCs must be 50-fold higher to attain similar densities. Therefore, to achieve the same level of staining as observed in our bead assay, over 175,000 Env183/A2 complexes would have to be presented. Hepatocytes, however, are poor antigen presenting cells with low expression levels of class I MHCs (79) and their capacity for antigen presentation is inefficient (227), making these specific viral pMHC products rare in a clinical setting. A caveat of this estimate for the lower limits of detection, is that it assumes random distribution over membrane,

whereas pMHC molecules could conceivably cluster (70, 100, 131). It therefore remains to be established if such clustering might allow binding by multivalent TCR constructs.

Collectively, we demonstrated the utility of the bead-based flowcytometry assay in conjunction with the peptide-exchange strategy for pMHC (11, 44, 73, 83, 85, 167, 199) to rapidly characterize the antigen-specificity and cross-reactivity of soluble TCRs and TCR-like mAbs, which is of significance especially when the two have demonstrably different docking orientations to their cognate pMHC (23, 134). Our results also underscore that antigen distribution is an essential parameter to be considered during the development and design of novel diagnostic and immunotherapeutic intervention strategies.

Figures

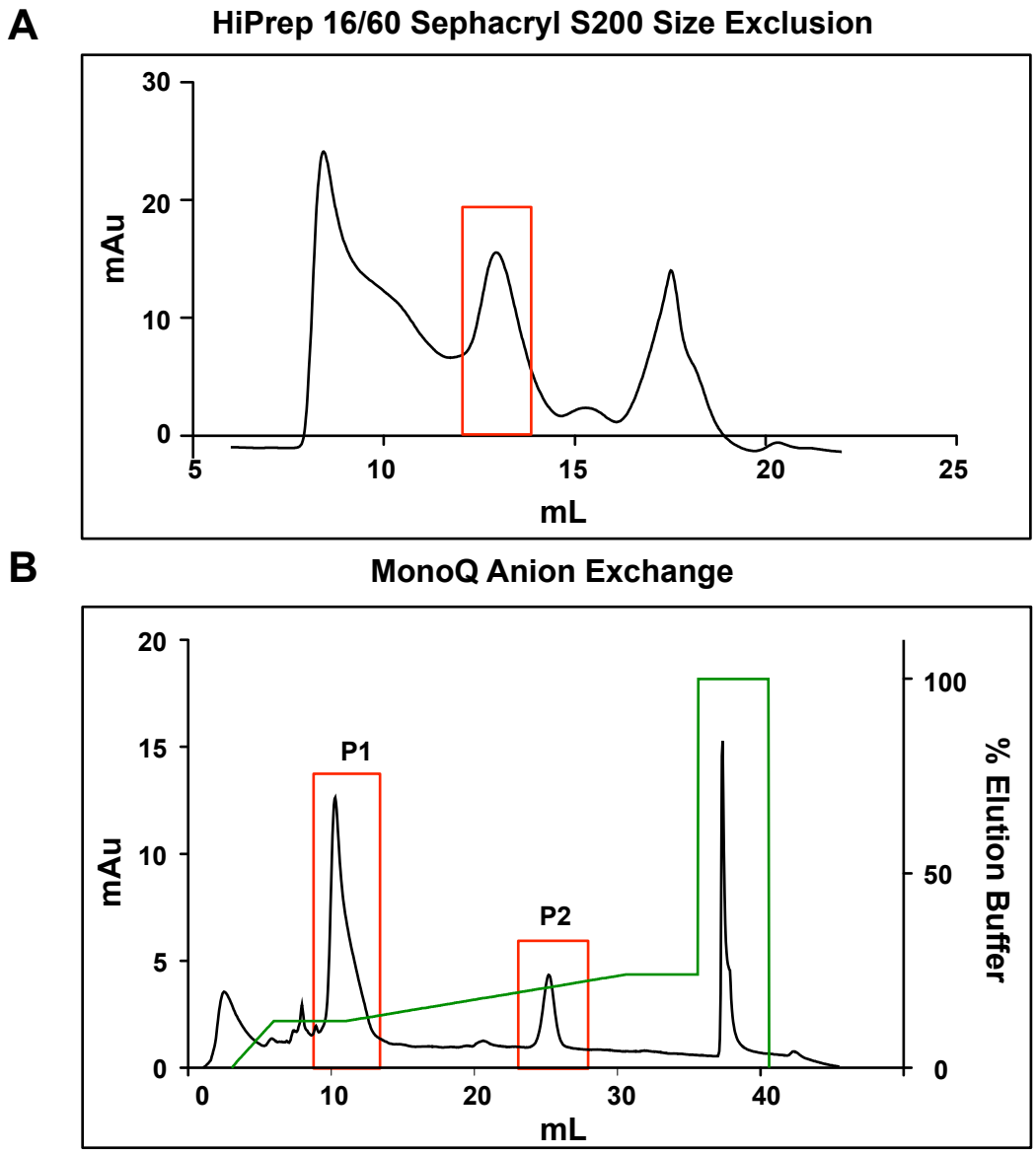


Figure 3.1. FPLC purification profiles of in vitro refolded soluble TCR. *In vitro* refolded TCR was purified by size exclusion chromatography (A) followed by anion exchange chromatography (B). Fractions collected, indicated in red boxes, from size exclusion chromatography were pooled and further purified by anion exchange chromatography. Two gradients were used for elution in the anion exchange purification step. A short 0-15% gradient was followed by a long 15-25% gradient of elution buffer (20mM Tris, pH 8.0 with 1M NaCl), replacing the binding buffer (20mM Tris, pH 8.0)

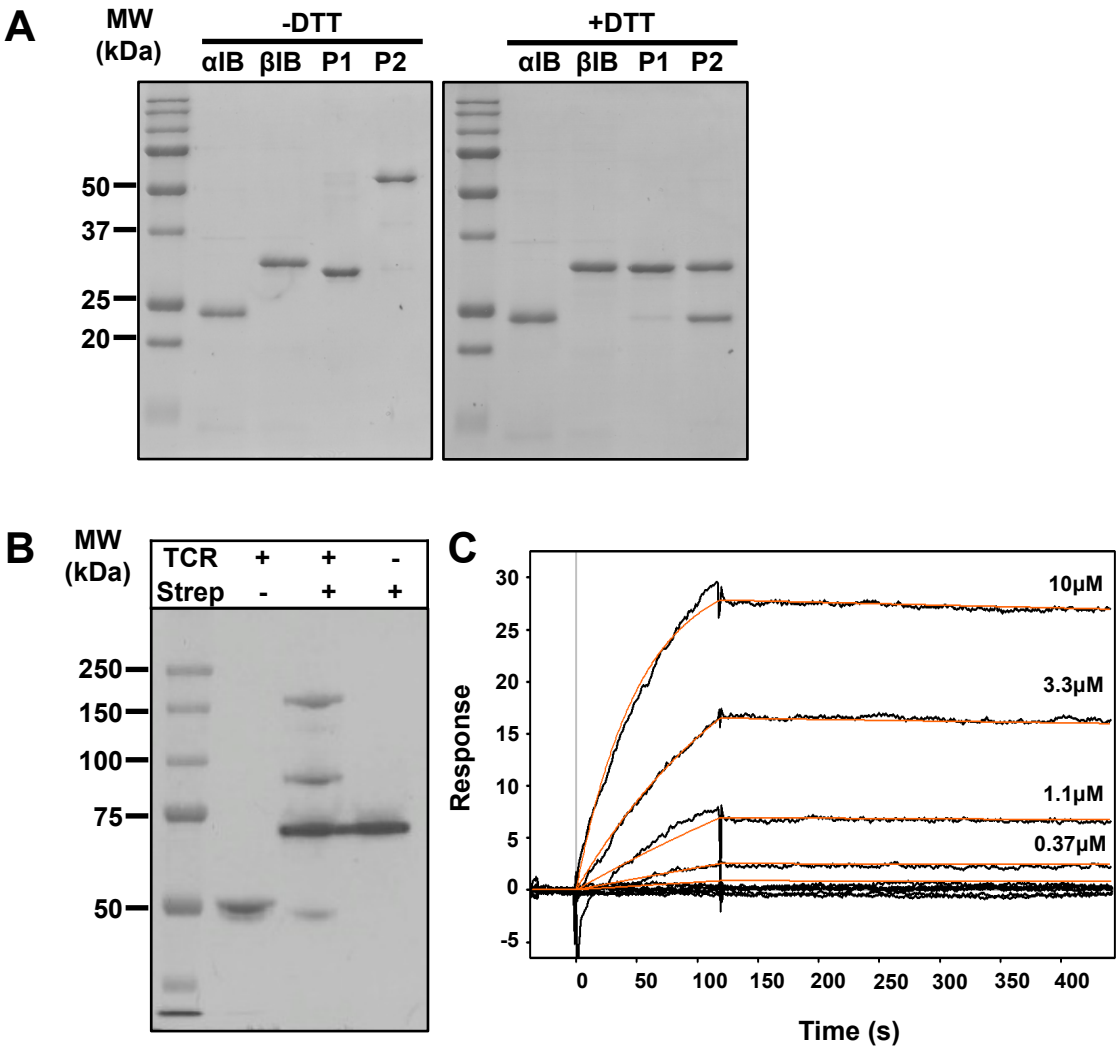


Figure 3.2. Protein analysis of purified soluble full length TCR. (A) Analysis of two distinct peaks obtained after ion exchange purification (Fig 1B) of refolded TCR showed that P1 contained predominantly monomers while P2 contained dimers. (B) Gel shift analysis of the refolded TCR product. The streptavidin Western blot showed biotinylation in Beta chain IB and refolded TCR. (-S, without streptavidin, +S, with excess streptavidin) and that >90% of the dimers were biotinylated. (C) Surface plasmon resonance analysis of refolded TCR tetramers demonstrated specificity for immobilized Env183/A2.

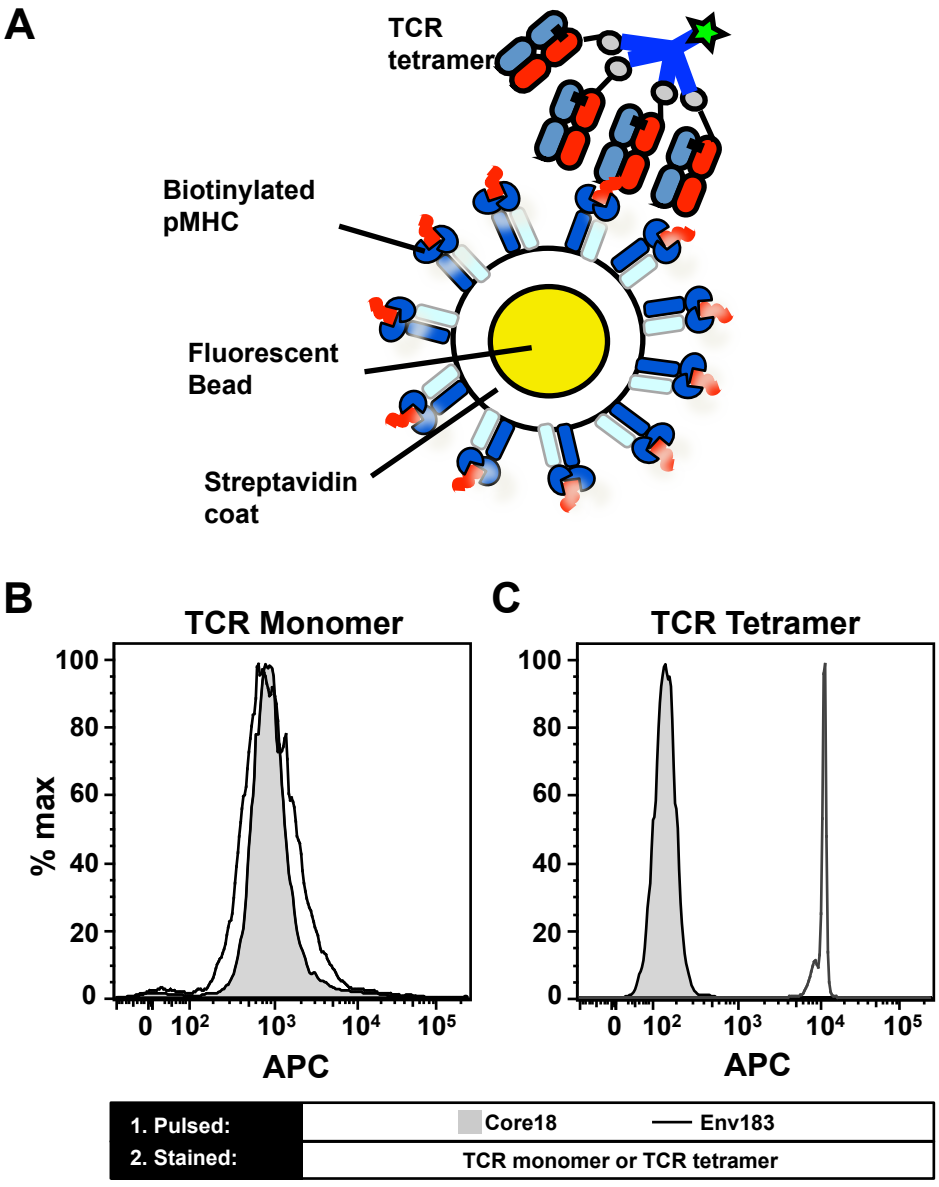


Figure 3.3. Peptide MHC complex coated beads as an artificial antigen presenting cell. (A) Schematic of the fluorescent bead assay. Streptavidin-coated beads were loaded with biotinylated pMHC monomer thus providing a homogenous binding surface for the Env183/A2 mAb or TCR tetramers (B) Beads were stained with 10µg/mL of monomeric TCRs or (C) 1µg/mL APC-conjugated TCR tetramers. Monomeric binding was probed using an anti-TCR antibody. Beads presenting $Core_{18\text{-}27}$ HLA-A*02:01 were used as a control.

A

Name	Sequence
Env183(A/C/D)	FLLTRILTI
Env183(B)	FLLTKILTI
Env355	WLSLLVPFV
Pol455	GLSRYVARL
Core18(A/D)	FLPSDFFPSV
Core18(B/C)	FLPSDFFPSI

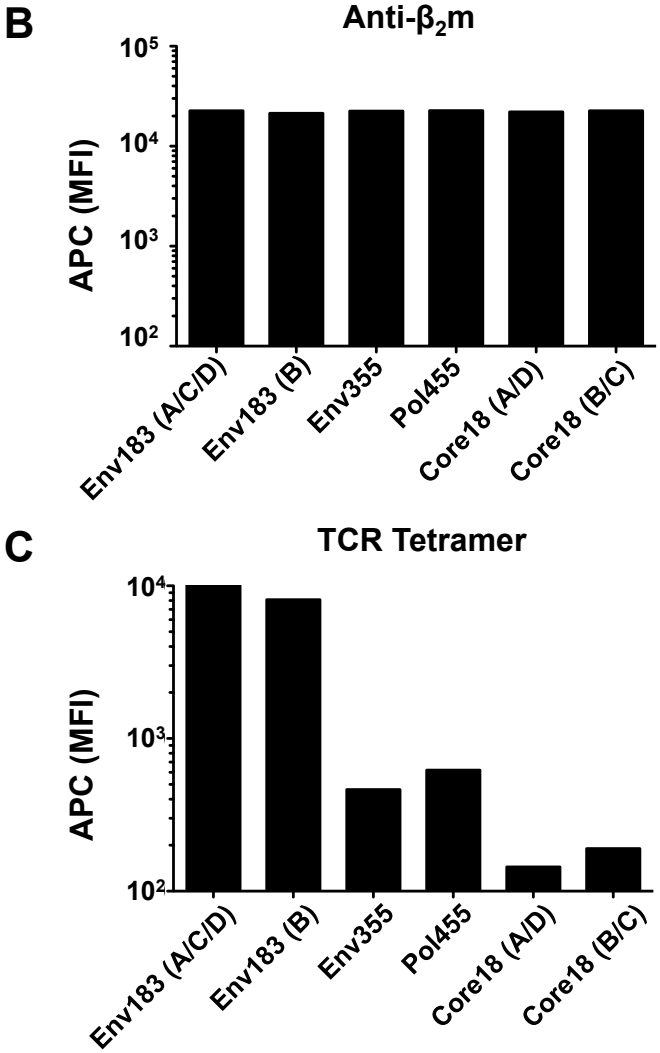


Figure 3.4. Soluble TCR binds specifically to $Env_{183-191}$ pMHC and recognizes both natural variants Genotypes A/C/D and Genotype B. (A) Beads were loaded with HLA-A*02:01 presenting 6 different peptides and stained with a mouse anti-β2m followed by detection with an APC-conjugated goat anti-mouse antibody. (B) Staining shows equal levels of pMHC on beads. (C) The beads were furthermore stained with 1µg/mL APC-conjugated TCR tetramers, showing that they only bound Genotype A/C/D and Genotype B variants of $Env_{183-191}$ peptides presented by HLA-A*02:01

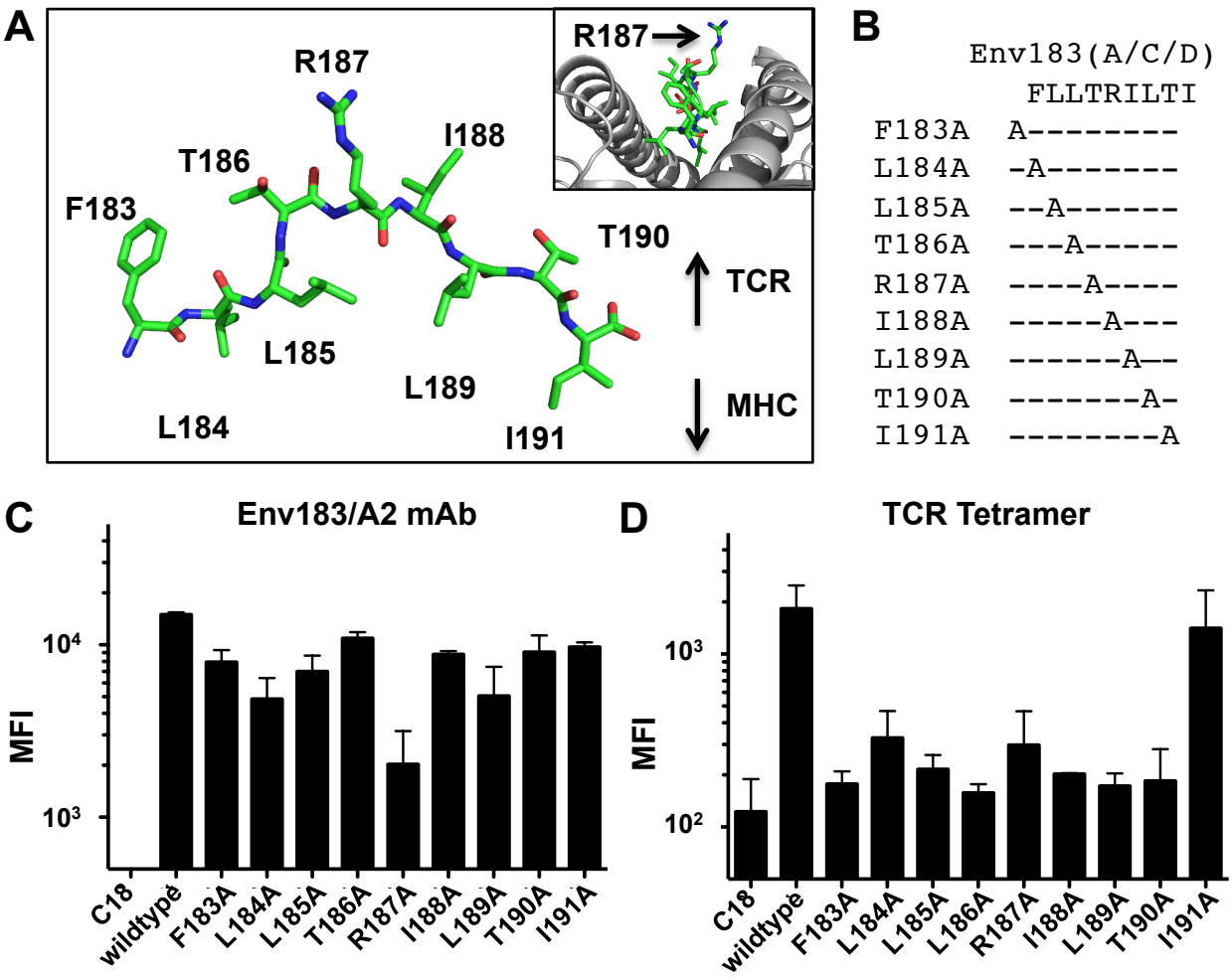


Figure 3.5. Comparison of fine specificity of Env183/A2 antibody and soluble TCR tetramers. (A) The $Env_{183-191}$ peptide modeled into the Tax/HLA-A2 crystal structure, inset: lateral view of the peptide in HLA-A*02:01. The Arg187 side chain protrudes from the binding pocket, potentially interacting with the TCR or antibody. (B) Nine different $Env_{183-191}$ peptides with single position alanine substitutions were used to probe the fine specificity. (C and D) The alanine variant pMHCs were loaded onto beads and stained with Env183/A2 mAb or TCR tetramer (1µg/mL).

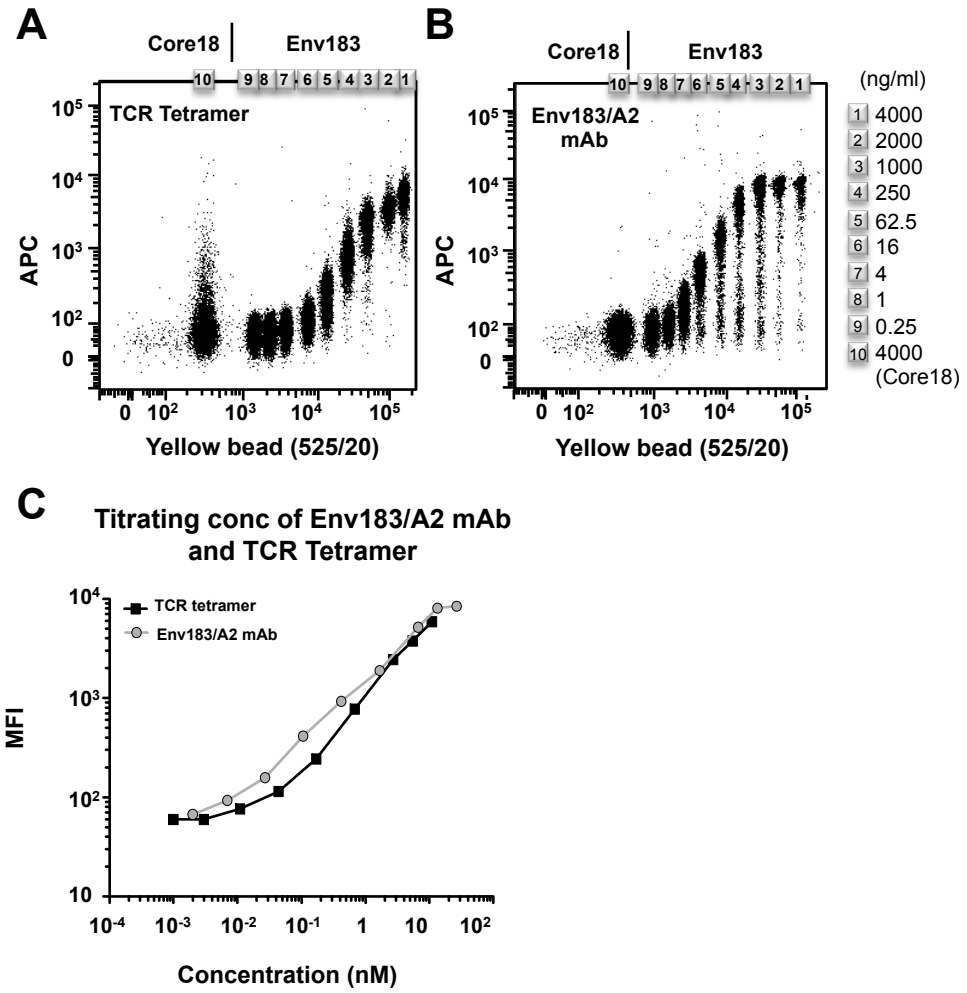


Figure 3.6. Functional avidity of the Env183/A2 antibody and soluble TCR tetramers. A range of beads of different fluorescent intensities were saturated with pMHC and stained with titrating amounts of (A) TCR tetramer or (B) Env183/A2 mAb. (C) The titration profiles of the TCR tetramer and the Env183/ A2 mAb showed that both reagents had similar avidity for the pMHC.

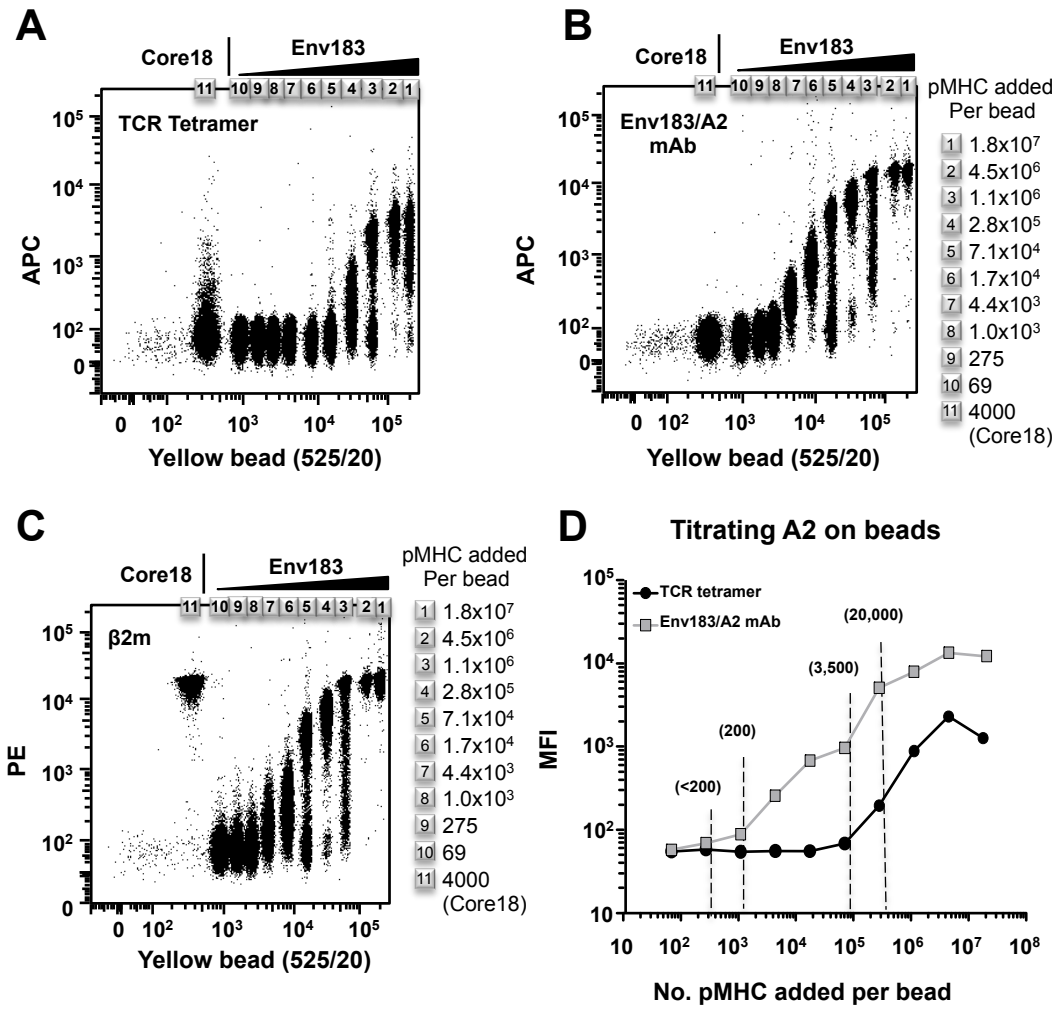


Figure 3.7. Peptide MHC sensitivity comparison of Env183/A2 antibody and soluble TCR tetramer. An array of beads presenting pMHCs at different densities was generated, as confirmed and quantified by anti-β2m staining. These beads were incubated with 1μg/mL of (A) TCR tetramer or (B) Env183/ A2 mAb. (C) Levels of pMHC presented on the surface of the beads used in (A) and (B) was determined by staining with a mouse anti-b2m antibody. (D) Graphical representation of staining intensity for the different bead populations. Whereas the antibody bound beads presenting 200 complexes, the TCR tetramer required at least 3500 complexes for similar staining.

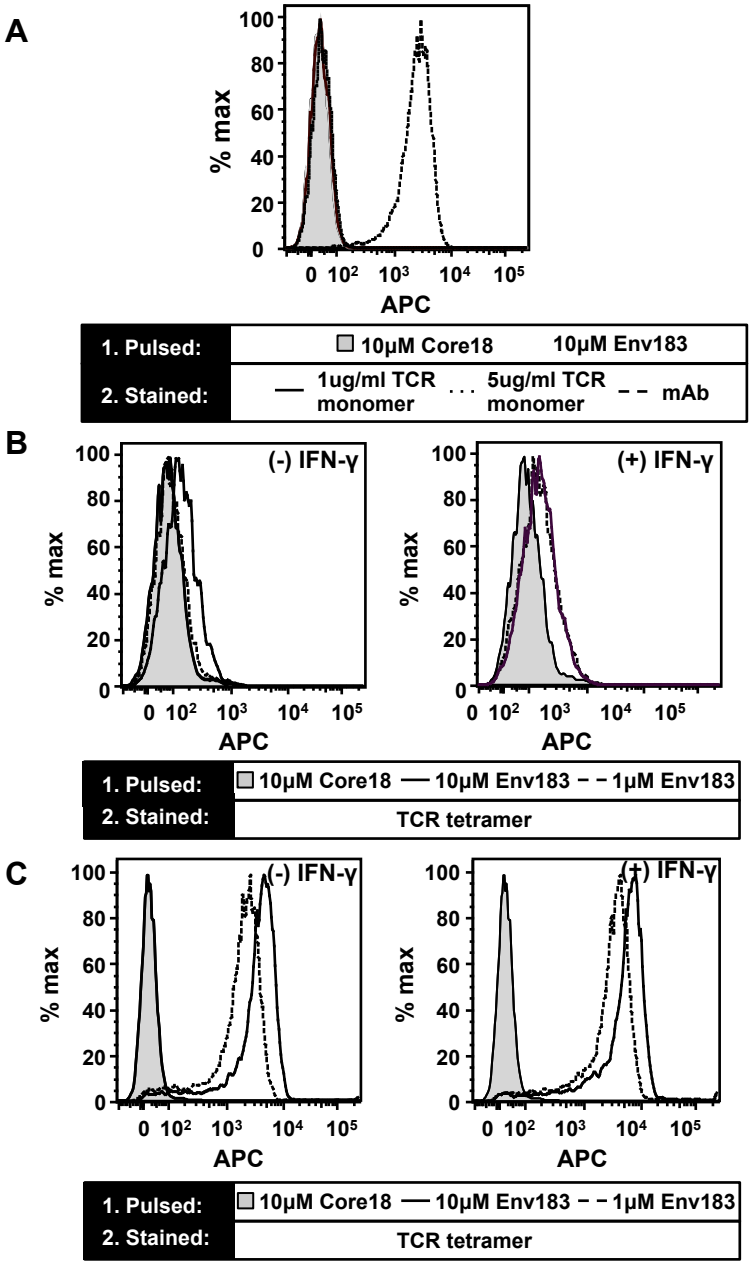


Figure 3.8. Recognition of exogenously pulsed pMHC presented on the surface of cells. (A) TCR monomers were used at 1μg/mL and 5μg/mL to stain T2 cells pulsed with 10μM $Env_{183-191}$ peptides. Binding of monomers was probed using an anti-αβTCR antibody. No significant staining of TCR monomers was seen. The E183/A2 monoclonal antibody was used as a positive control. (A) Untreated (-) and T2 cells treated (+) with IFN-γ were pulsed with 1 or 10μM of $Env_{183-191}$ or 10μM of $Core_{18-27}$ peptide control and stained with 1μg/mL TCR tetramer. (B) Identically treated T2 cells stained with 1μg/mL Env183/A2 mAb demonstrates that both treatment with IFN-γ and pulsing with higher peptide concentrations results in a higher surface expression of Env183/A2 complexes. .

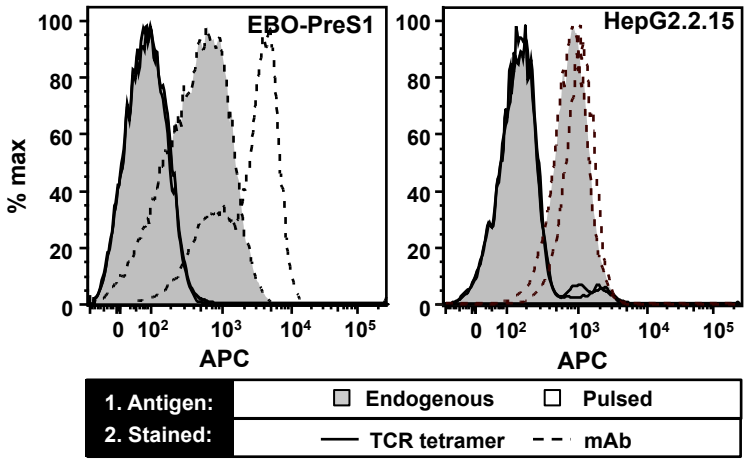


Figure 3.9. Recognition of endogenously presented pMHC complexes. HepG2.2.15 and EBO-PreS1 cells, transfected with the full and PreS1 fragment of the HBV genome, respectively, endogenously process and present Env183/A2 pMHC complexes on their cell surface. The TCR-like mAb (dashed) shows improved detection compared to the TCR-tetramers (solid). Peptide pulsed HepG2.2.15 and EBO-PreS1 further increased surface levels of Env183/A2 (filled histograms). TCR tetramers did not give significant staining with unpulsed or pulsed cells.

Dilution Factor	pMHC added per bead	Thereotical no. of beads conjugated per bead	MFI of β2m staining	No. of Complexes by Qifi™ Kit
No dilution	1.81×10^7	2.268×10^6	18694	78789
1 in 4	4.52×10^6	2.268×10^6	18522	78021
1 in 16	1.13×10^6	1.13×10^6	9153	36943
1 in 64	2.82×10^5	2.82×10^5	5315	20757
1 in 256	7.05×10^4	7.05×10^4	996	3514
1 in 1024	1.76×10^4	1.76×10^4	246	797
1 in 4096	4406	4406	133	415
1 in 16384	1101	1101	63.5	190
1 in 65536	275	275	64	191
1 in 262144	69	69	57.8	172

Table 3.1. Quantification of pMHCs presented on streptavidin-coated beads. The QIFI™ quantification kit contains 5 beads conjugated with known number of mouse IgG. IgG. The beads were stained with an APC-conjugated goat anti-mouse and the mean fluorescent intensity (MFI) of each bead population was recorded. A linear regression was determined between the log(MFI) and log(no. of IgG) and the parameters were used for later calculations of pMHC numbers on the streptavidin beads. Beads stained with a mouse anti-β2m antibody (Figure 3.10C) and subsequently with the same APC-conjugated goat anti-mouse antibody used to stain the QIFI™ beads and the number of pMHC complexes were calculated based on the mean fluorescence intensity of each anti-b2m staining.

CHAPTER FOUR

DEVELOPMENT OF PEPTIDE-MHC-COATED BEADS FOR DETECTION OF T CELL RECEPTORS IN A YEAST DISPLAY SYSTEM

Introduction

The T cell receptor (TCRs) binds to an antigenic peptide presented on the cell surface by a protein encoded by the major histocompatibility complex (MHC) with low affinities (K_D values between 1-100μM) (112). This recognition process is also challenging because the number of specific peptide-MHC complexes is usually low, as few as about 10 per target cell (226). These factors limit the application of soluble versions of the TCR as a peptide-MHC complex (pMHC) targeting tool. However, the ability to engineer proteins for improved binding has developed over the past years. Accordingly, various systems including phage display, yeast display, mammalian cell display, insect cell display and ribosome display have been used as a platform for the expression and directed evolution of mutant proteins (25, 29, 41, 118, 128). Each system has its advantages and disadvantages. For example, while mammalian cells might be preferred for the expression and display of membrane proteins such as the TCR, the library diversity is limited by the efficiency of retroviral transduction and the number of cells that can be handled. Yeast and phage display systems allow libraries with diversity orders of magnitudes larger than mammalian cell systems, but expression and display of stable proteins may be hampered by the lack of the required post-translational modifications or other cellular machinery. Nevertheless, several groups

have reported success using the yeast, phage and mammalian display systems in the engineering of TCRs (41, 95, 118).

The antigen binding site of a TCR consists of hypervariable loops in the α and β variable domains, known as the complementarity determining regions (CDRs). Each variable domain contributes three CDRs with a total of six CDRs determining the specificity and affinity of the TCR. Importantly, CDR3 loops of each variable domain are encoded by the junctions (VJ or VDJ) at RAG recombination site. Thus, these loops show the most extensive diversity in sequence and length. As the TCR docks diagonally over the pMHC ligand, both CDR3 loops are positioned primarily over the peptide while the CDR 1 and 2 loops are focused primarily on the binding of the helices of the MHC molecule (76, 112, 170). Hence, diversity of CDR3 loops in TCRs play a major role in conferring peptide specificity; accordingly, to retain peptide specificity, the CDR3 loops are an attractive target for mutagenesis and affinity maturation.

The mouse 2C TCR was the first TCR to be engineered for higher affinity, using the yeast display system. The 2C TCR recognizes a peptide called QL9 peptide restricted by the allogeneic MHC class I molecule L^d and the foreign peptide called SIY presented by the syngeneic MHC class I molecule K^b. The 2C TCR has been expressed in a single chain, Vβ-linker-Vα, form (scTCR) and higher affinity mutants have been isolated by mutating amino acid residues in the CDR3α and CDR3β regions (47, 95). Similarly, the human A6 TCR specific for the human T-lymphotropic virus (HTLV) Tax peptide restricted by the human MHC class I molecule HLA-A2 and the 1G4 TCR specific for tumor-associated peptide antigen, NY-ESO-1 also restricted by HLA-A2 had been engineered with mutations in the CDR3β and CDR3α regions (118). In addition, it

has also been shown that affinity can be improved with mutations in the CDR1 and CDR2 regions (47, 61, 118). Thus, all six CDRs are potential candidates for consideration when designing mutant libraries.

The number of residues to be mutated, and hence the overall theoretical diversity, is limited by the size of the library achievable by the display system. For example, a library containing 5 randomized amino acid positions would theoretically contain $3.2x10^6$ possible amino acid sequences. Employing the NNS codon degeneracy (where N: adenine, cytosine, guanine or thymine; S: guanine or thymine), covering all 20 amino acids with 32 possible codons, the library would theoretically contain $3.3x10^7$ possible nucleotide sequences. To cover all possible sequence space also requires oversampling due to probability issues and PCR or codon synthesis biases. There are also limitations imposed by transformation efficiency (10^7-10^8 transformants/μg plasmid in yeast display (14, 165)) during the generation of the library, the sensitivity and efficiency of analytical techniques used for the screening of the library (e.g. fluorescence-activated cell sorting) and other practical issues such as time and cost. Hence, library design and screening approaches will influence the success of engineering an affinity-matured protein.

In the yeast display system, TCRs have been screened with fluorescent-labelled, soluble forms of pMHC ligands, often in the form of immunoglobulin-linked dimers or streptavidin conjugated-tetramers, followed by fluorescence-activated cell sorting (FACS). Yeast displayed libraries are typically subjected to repeated rounds of cell sorting with successive reduction in ligand concentrations. This increases the stringency of selection during each sort, creating the selective pressure required to isolate mutated

cloned with the highest affinity. Ideally, a mutant library covering all six CDRs would provide the diversity required to isolate the best solutions for affinity-matured variants. However, this would involve the construction of library sizes that are not achievable. The availability of structure-activity data can enable a semi-rational design of libraries in which key residues are selected for mutagenesis, thereby reducing the required library size. Unfortunately, structural studies of TCRs have been less successful than antibodies, largely due to the difficulty in producing soluble TCRs (169).

This chapter presents studies based on the premise that better selections strategies would be capable of isolating higher affinity TCR mutants, even if the library sizes, and thus sequence space, are not optimal. Specifically, it examines whether the multivalent bead approach used in Chapter 3 would facilitate the isolation of higher affinity TCR mutants from yeast display libraries.

It has now been over 15 years since the first use of multimeric pMHC molecules enabled the detection and study of antigen specific T cells (6, 54). The avidity provided by multimeric pMHC prolonged the fast dissociation rates associated with the weak TCR:pMHC interaction, allowing for detection by flow cytometry. Similarly, dimeric and tetrameric pMHC had also been employed in the screening of yeast displayed TCR libraries (47, 94, 95). In this chapter, I present a strategy to screen yeast displayed TCR libraries with sensitivity that is even greater than dimeric and tetrameric pMHC, by employing the use of streptavidin-coated beads. Biotinylated pMHCs were immobilized on the surface of the streptavidin-coated beads at high densities, to provide a detection reagent with higher sensitivity than achievable with dimers or tetramers. To quantitatively examine the system, yeast displaying the single-chain 2C TCR with wild

type affinity and the higher affinity variants called Y48A and m33 that bind the SIY/K^b pMHC with K_D values of 30μM, 3μM and 30nM, respectively, were used. Using these model TCRs, I showed that the multivalent pMHC beads have greater sensitivity than conventional dimeric and tetrameric reagents. To apply the approach to a TCR library of mutants, I used the pMHCs beads to screen a *de novo* library, isolating both high and intermediate affinity TCR variants. Only the high affinity variants were isolated using the less sensitive reagents such as pMHC tetramers and dimers. In summary, I present a new strategy in library screening whereby the use of multivalent pMHC beads allowed the identification of both intermediate and higher affinity mutants in a TCR library. The approach will be useful in the case of protein engineering in which libraries lack the highest affinity solutions due to constraints in the design or construction of the library. Such intermediate affinity solutions can then serve as the leads for additional library construction and affinity improvements.

Materials and Methods

Yeast display of single-chain and full-length T cell receptors

The single-chain form of the wild type 2C TCR, 2CT7 was engineered previously for yeast surface expression (108) while the high affinity mutant m33 was generated by directed evolution in a yeast display system (94). The Y48A mutant is a single-residue variant of the m33 mutant in the CDR2β region ($Y48_{\beta}A$) (43). The single-chain form of the Tax-HLA-A*02:01 (Tax/A2) specific A6 TCR (X15) was previously generated by random mutagenesis in a yeast display system (2). All scTCRs were cloned in the yeast display vector pCT302, and expressed on the surface of yeast as a fusion to the cell

wall protein Aga2. EBY100 yeast cells were transformed with the scTCR containing yeast display plasmids and cultured in Trp^- media at 30°C for selection. Expression of the TCR genes was induced by transfer to galactose containing media and cultured at 20°C.

Full-length β-chain (VβCβ) of the HBV Core18/HLA-A*02:01 specific TCR was cloned into the yeast display, Aga2 plasmid p315 while the α-chain was cloned into the pCT302-NAGA yeast secretion plasmid. The p315 plasmid contains a leucine biosynthetic selectable marker that allows transformants to be cultured in Leu^- media while the pCT302-NAGA plasmid contains a tryptophan biosynthetic selectable marker that allows transformants to be cultured in Trp^- media. Double transformants were selected in Leu^--Trp^- selection media. Induction of expression of the TCR genes from both plasmids was done in galactose containing media at 20°C.

In vitro refold and biotinylation of pMHC complexes

Heavy chain and β2-microglobulin (β2m) subunits of the mouse K^b and human HLA-A2 class I MHC molecules were expressed individually in BL21 *E. coli* cells. Inclusion bodies were harvested after induction of BL21 cultures and purified before refolding *in vitro* with the appropriate peptides. Refolding was done in refold buffer (100mM Tris pH8.0, 400mM L-Arg HCl, 2mM EDTA) supplemented with 0.5mM oxidised glutathione and 5mM reduced glutathione in the presence of excess peptides at 4°C. Heavy chain and β2m inclusion bodies were introduced in three separate doses 8-12 hours apart and allowed to refold before purification by size exclusion chromatography. In vitro biotinylation was done enzymatically using biotin ligase

according to manufacturer's instructions (Avidity, LLC) and biotinylation of the refolded pMHCs was verified by gel-shift with streptavidin by SDS-PAGE (Figure 4.1A).

Production of and yeast cell staining with pMHC beads

Blue (SVBP-03-10) and yellow (SVFP-0552-5) fluorescent streptavidin-coated beads (Spherotech Inc) beads were first pre-incubated with 200µL of wash buffer (2% BSA in PBS) for 30 minutes with shaking and then incubated in with pMHC in wash buffer for 30 minutes with shaking. Binding capacity of the beads was calculated based on the manufacturer's specifications. pMHC-loaded beads were washed with blocking buffer and suspended in wash buffer to the required concentration. Immobilization of pMHC on the surface of beads was probed with an anti-β2m antibody and analysed by flow cytometry (Figure 4.1B). Yeast cells were stained with pMHC beads at room temperature for 30 minutes with shaking. Stained cells were washed twice with wash buffer before flow cytometric analysis.

Fluorescence-activated cell sorting of de novo library

The *de novo* scTCR library was designed and provided by Sheena Smith. The library, which contained degeneracy at 5 selected amino acids, was synthesized by Genescript, and cloned into the pCT302 yeast display vector. Yeast cells transformed with the yeast library were grown in Trp^- media for selection and induced for TCR expression before sorting.

Streptavidin beads were supplied in storage buffer containing 0.02% sodium azide (w/v) to prevent microbial contamination. To assess toxicity and sterility of the

beads, they were first washed thoroughly in excess sterile washing buffer and added to growing yeast culture. No contamination or toxicity was observed.

In general, 10^7 cells were stained with 10μg/mL (with respect to pMHC) of pMHC-coated blue fluorescent beads for 30 mins at room temperature. After incubation, the cells were washed and suspended in washing buffer before sorting. The top 1% (0.5% for the final 2 sorts) of cells exhibiting specific binding by detection of blue fluorescence in the APC channel were isolated and collected using a BD FACSAria fluorescence-activated cell sorter. Collected cells were expanded in Trp^- media before subsequent induction and sorting. Cells collected after 4 sorts were expanded in culture and plated on Trp^- media for the isolation of single clones that were analyzed for binding. The pCT302 yeast display vectors of individual clones were recovered using the Zymoprep kit (Zymo Research) according to manufacturer's instructions and amplified by PCR before submission for DNA sequencing.

Results

Production of pMHC-coated beads

The murine TCR 2C binds the peptide SIY (SIYRYYGL) in complex with the class I molecule K^b (SIY/K^b), while the human A6 TCR recognizes the human T-cell leukemia virus (HTLV) Tax11-19 peptide in complex with the class I molecule HLA-A*02:01 (Tax/A2) (201, 204). To evaluate the use of the streptavidin-coasted beads as a scaffold for pMHC presentation, recombinant soluble SIY/K^b and Tax/A2 pMHC were refolded in vitro, purified and biotinylated. Biotinylation of the refolded products were probed by a streptavidin gel shift assay using SDS-PAGE. Figure 4.1A shows that at

least 50% of the refolded pMHCs were biotinylated. The biotinylated pMHC were then used to generate the multivalent pMHC coated-beads. The binding capacities of the streptavidin-coated beads were calculated based on technical specifications provided by the manufacturers. The concentration of pMHCs used for immobilization was then based on achieving 5-fold excess over the binding capacity of the beads, in order to ensure saturation. The densities of pMHC on the surface of the beads were probed using an antibody specific for the β2-microglobulin (β2m) subunit of the pMHC complexes. Figure 4.1C shows that when a titration of pMHC concentrations is used during incubation, ranging from 5:1 to 0.0005:1 pMHC to streptavidin binding site ratio, beads conjugated with various densities of pMHC can be made and that 5-fold excess of pMHC was sufficient to achieve saturation.

pMHC beads have higher sensitivity than dimeric or tetrameric pMHCs

The current "gold standard" for analyzing T cells or isolating yeast-displayed TCR mutants are multimeric pepMHC ligands produced with Ig-fusions or streptavidin. To examine the use of multimeric beads, I used the 2C yeast display system. Wild-type 2C TCR binds to SIY/K^b pMHC with a K_D value of 30μM, while the high-affinity m33 mutant binds 1000-fold better with a K_D value of 30nM. The single-amino acid mutant of m33, called Y48A, binds with an intermediate affinity of 3μM (43, 78). Collectively, these provided a useful system with a wide range of affinities to explore the limits of binding by the various multimeric forms of SIY/K^b.

Yeast cells expressing the single-chain stabilized form of the wild type 2C receptor (called 2CT7), m33 and Y48A were induced and cells were stained with

dimeric SIY/K^b (DimerX, an immunoglobulin fusion of K^b), SIY/K^b tetramers of streptavidin, and SIY/K^b-coated beads. The concentration used of each reagent was fixed with respect to SIY/K^b molecules at 10μg/mL (200nM). Dimeric and tetrameric forms of the SIY/K^b pMHC were able to stain only the highest affinity mutant m33, but not 2CT7 of wild type affinity or the Y48A mutant with intermediate affinity (Figure 4.2A and B)(Note: negative populations, as seen in the staining of m33, are invariably observed in all yeast-displayed systems as a result of cells that have lost plasmid during induction; this population serves as an internal negative control). The ability to stain m33 but not 2C or Y48A with tetramers had also been shown previously (43). In striking contrast, the SIY/K^b-coated beads were able to bind both the high and intermediate affinity receptors, and also had modest staining of the low-affinity wild type TCR, 2CT7 (Figure 4.2C), as compared to the background staining with the control OVA/K^b-coated beads (Figure 4.2D). SIY/K^b-coated beads staining of the highest affinity TCR m33 also exhibited fluorescence intensity that was almost ten-times that of the dimer or tetramers, providing a system with further enhanced sensitivity.

The sensitivity of the pMHC-coated beads was further tested with the use of variants of the SIY peptide (SIYRYYGL) with known effects on 2C and m33 binding. The structure of 2C-SIY/K^b shows that both the side chains of the Arg residue at position 4 (R4) and the Tyr residue at position 6 (Y6) point toward the 2C TCR (Figure 4.3A). T cell-activation assays were done with 2C TCR transfectants, showing that levels of IL-2 release were affected when alanine substitutions were made at these positions of the SIY peptide, even when antigen-presenting cells were pulsed with high concentrations of peptides (100μM) (31). In addition, a TCR mutant (m67) similar to

m33 showed about 100-fold lower affinities for these two SIY variants, bound to K^b (31). To look at the effect of these substitutions on TCR binding, recombinant SIY(R4A)/K^b and SIY(Y6A)/K^b were refolded in vitro, immobilized on streptavidin beads (as verified with anti-β2m staining, Figure 4.2B), and used to stain yeast cells displaying the 2CT7, Y48A and m33 TCRs. Both peptide variants significantly reduced binding to Y48A and m33 TCRs. The R4A variant had a more profound effect compared to Y6A as binding was reduced to levels equivalent to that observed with the beads conjugated with the non-cognate OVA/K^b pMHC control (Figure 4.2D). The effects of these SIY peptide variants on binding to the wild type 2C receptor were previously untested due to limitations of detection when using tetrameric pMHCs or soluble 2CT7 scTCRs (31, 43). However, with pMHC-coated beads, the Y6A substitution is observed to result in a modest reduction in binding to the wild-type 2C receptor as compared to its effect on binding to the higher affinity mutants (Figure 4D), while the R4A substitution had a larger effect on binding to the TCR (Figure 4E). These results also suggest that the sensitivity of the pMHC beads is over 100-fold improved compared to dimers and tetramers, with a sensitivity of at least a K_D value of 30μM.

Sensitivity of pMHC staining varies with beads concentration and pMHC density.

During the screening of a yeast-displayed TCR library, stringency of selection can be increased after each rounds of sorting by decreasing the concentration of pMHC used during staining. With the pMHC beads, the concentration of pMHC can be varied in two different ways. First, assuming each bead is saturated according to the calculated binding capacity, the concentration of pMHC can be approximated by the number of

beads used for staining. A titration of SIY/K^b beads, representing pMHC concentrations of 1ng/mL to 10μg/mL, was thus used to stain yeast cells expressing 2CT7, Y48A and m33 TCRs on their surface. Figure 4.4 shows that the binding of the three TCRs to the SIY/K^b beads gradually decreases when lower concentrations of SIY/K^b, were used. Specific binding of low affinity wild-type 2CT7 TCR to SIY/K^b beads as compared to the control OVA/K^b beads was detectable even when the pMHC concentration was as low as 100ng/mL (2nM).

Another approach to varying the pMHC density on beads is to titrate the concentration of pMHC used during bead preparation, at a fixed number of beads. As shown in Figure 4.1C, saturation of beads was observed when a 5-fold excess of pMHC, above the number of biotin binding sites, was used. To generate beads presenting different densities, the amount of pMHC used was titrated in 10-fold dilutions. Excess streptavidin binding sites were blocked with free biotin, and the density of conjugation was probed by staining with an anti-β2m antibody Figure 4.1C. Equal amount of the beads were then used to stain yeast cells displaying the 2CT7, Y48A and m33 TCRs. These results showed that only the two highest concentrations of SIY/K^b used, when the beads are fully saturated (Figure 4.1C), were capable of detecting the 2C and Y48A TCRs. Below these densities the beads presumably have insufficient avidity for specific binding and retention during flow cytometry. In contrast, the m33 TCR yields staining at ten-fold lower densities, consistent with the ability of pMHC dimers or tetramers to detect this TCR. In summary, the density of pMHC in beads can be tuned to achieve the selection of TCRs within a desired range of affinities, to affinities as low as 30 μM.

Selection of TCRs with intermediate and high affinities from a scTCR library with pMHC beads

Having demonstrated the ability of the beads to bind low affinity scTCRs displayed on the surface of yeast, I next tested whether the beads could be used to screen a scTCR library. A proprietary library based on the A6 TCR, specific for the HTLV Tax peptide restricted by the human MHC class I molecule HLA-A*02:01 (Tax/A2), was designed and generated by Sheena Smith. Recombinant HLA-A*02:01 complexes with the Tax peptide, and a control hepatitis B virus peptide $Core_{18-27}$ (C18/A2) were refolded in vitro and purified. These pMHC were prepared in sterile conditions and used in saturation (10µg/mL of pMHC) to coat streptavidin beads. The Tax/A2 and C18/A2 beads were used to stain the unsorted A6 library (Figure 4.5A). The top 1% of cells that stained positive were subsequently sorted and expanded in culture media. Three further rounds of sorting were done with the top 1% (2^{nd} round) and 0.5% (3^{rd} and 4^{th} rounds) of binders collected and cultured. Figure 4.5B summarizes the binding profiles of the cells collected after each round of sorting. After 4 rounds of sorting, a population of yeast cells that bound to Tax/A2 beads strongly was enriched, whereas no such population was isolated in the sorts using the C18/A2. This suggests that the population enriched was specific for the cognate Tax/A2 pMHC and not due to non-specific binding to the bead scaffold.

Isolation and characterization of Tax/A2 binding clones

Yeast cells collected from the 4^{th} sort of the Tax/A2 selections were plated and individual clones were picked for further characterization. The clones were analyzed in

comparison to the single-chain, stabilized, high-affinity mutant X15 (2). Twenty individual clones were induced for expression, and then stained with either Tax/A2 beads or the control C18/A2 beads, at the pMHC concentration used for sorting. For comparison, the clones were also stained with Tax/A2 and C18/A2 tetramers at the same pMHC concentrations.

The A6 X15 TCR is the single-chain stabilized form of the A6 mutant clone 134, which was engineered for high-affinity (K_D value of 2.5nM) previously by phage display (2, 118). Figure 4.6A shows the binding histograms of yeast-displayed X15, stained with either Tax/A2 beads or Tax/A2 tetramers. As with the m33 high-affinity TCR, both the beads and tetramers were capable of staining X15, although the beads yielded enhanced fluorescence intensity. In comparison to X15, two different classes of clones were identified from enriched cells (Figure 4B): 1) Yeast-displayed TCR mutants that bound strongly to both Tax/A2 beads and Tax/A2 tetramers (designated as strong binders) and 2) Yeast-displayed TCR mutants that bound to Tax/A2 beads but bound poorly or not at all to Tax/A2 tetramers (designated as intermediate binders). Figure 4.6B shows the binding profiles of 3 examples of each class of TCRs. Again, in comparison to the staining profiles of the three 2C TCRs (Figure 4.2), the strong binders are similar to the high-affinity m33 while the intermediate binders more closely resemble the Y48A mutant.

Three strong binders and three intermediate binders were further characterized by performing a titration with Tax/A2 beads and Tax/A2 tetramers. The titration profiles of the strong binders showed that they bound similarly (H4-1 and H4-11) or stronger (H4-3) than the high affinity X15 clone (Figure 4.7A), while they were negative for

staining with the control C18/A2 beads and tetramers. The titration profiles of the intermediate binders showed reduced binding to both Tax/A2 beads and Tax/A2 tetramers in comparison to clone X15 (note the y-axes for the intermediate binders differs from the strong binders by several fold). There was some specific binding at the highest concentrations of tetramers tested. In addition, sequences of the yeast display plasmids recovered from the strong and intermediate binders showed yielded unique sequences (data not shown). These findings support the idea that the pMHC bead-based approach can yield TCR mutants that are likely to have a wider range of affinities than can be isolated with pMHC tetramers. The expression of soluble forms of the scTCRs and their analyses by surface plasmon resonance will be required to confirm this finding and to determine the range of affinities associated with the mutants.

Screening for HBV C18/A2 specific TCRs from a full-length TCR library with pMHC beads

TCRs engineering by yeast display has been done previously with single chain forms of TCRs expressed as a Vβ-linker-Vα fusion protein on the surface of the yeast cell. However, these truncated TCRs are not stable and require additional mutagenesis to isolate stabilized forms of the single-chain fusion protein (2, 163). An alternative to expressing the Vα-linker-Vβ form is to display full-length receptors (VαCα and VβCβ), excluding the stalk and intracellular regions, on the surface of yeast cells. While such a strategy has been used in the engineering of high-affinity TCRs by phage display system (61, 115), it has not been used in the yeast display system. Toward this effort, here I cloned the genes for a TCR specific for the hepatitis B $Core_{18-27}$/HLA-A*02:01

(C18/A2) into the yeast display system, and I attempted to use the pMHC beads strategy to isolate affinity-matured mutants from full-length CDR3α-libraries of the TCR.

Single transformants expressing yeast-displayed β-chain of the C18/A2 TCR were first generated by transforming EBY100 yeast cells with the p315 yeast display vector containing the full-length VβCβ gene (Figure 4.8A). Transformants were screened for expression of the β chain using an anti-human TCR Vβ8.2 monoclonal antibody specific for the C18/A2 TCRβ chain and the highest expressing yeast cells were used for a second transformation with the pCT302-NAGA secretion plasmid containing the VαCα gene. Due to the lack of an antibody specific for the Vα3 of the C18/A2 TCRα chain, a c-Myc epitope tag was added to the C-terminal end of the VαCα gene as a probe for expression (Figure 4.8A). To facilitate the association of the secreted α chain and yeast displayed β chains, a non-native disulfide bond was engineered in the C regions, as described previously (30). Figure 4.8B shows the level of display, and presumably association, of the α and β chains on the surface of double transformants. These cells were incubated with the C18/A2 beads but staining above the background was not observed. To determine if this might be due to the relatively low fraction of cells that expressed the α chain, the sample was sorted with anti-c-Myc antibody. Sorting of the pCT302-NAGA-VαCα plasmid transformed cells with the anti-c-Myc antibody yielded a population that was enriched for expression of the c-Myc-containing α chain. However, these cells were not positive for staining with C18/A2 beads. These results indicate that either the α and β chains are not properly associated on the yeast surface, or the affinity of this wild-type TCR is very low.

To examine if a higher affinity mutant might be isolated, two libraries with 4-codon amino acid degeneracy in the CDR3α the C18/A2 TCR were produced (Table 4.1). PCR products containing the degeneracies were co-transformed with the linearized vector into yeast cells to generate the two full-length CDR3α libraries (Lα1 and Lα2). Lα1 and Lα2 were first subjected to a round of sorting based on c-Myc staining to enrich for α chain expression (Figure 4.8C). These populations were cultured, induced, and incubated with C18/A2 beads at a pMHC concentration of 10μg/mL. Four rounds of sorting was performed, with the top 1% of binders collected during the first two rounds and the top 0.5% of binders collected in the 3rd and 4th rounds. However, no enrichment of yeast mutants that bound to C18/A2 beads was observed after four sorts (Figure 4.8D).

Possible explanations for the failure to enrich affinity matured pMHC binders are: 1) the level of properly associated αβ heterodimeric TCR on the surface of the yeast is low, even though both chains were detectable, 2) the CDR3a libraries produced did not contain a high-affinity “solution”. In regard to the first possibility, as shown in Chapter 3, the efficiency of a multivalent reagent is dependent on the density of the target since the benefit of multivalency is the simultaneous binding of the multiple ligands and thus prolonging the off-rate (54). In the full-length system, the functional heterodimeric TCR is formed by the yeast displayed β chain and the secreted α chain. The yeast cells expressing either the wild-type C18/A2 TCR (Figure 4.8B) or the CDR3α libraries (Figure 4.8C) expressed high levels of expression of the β chain, similar those seen in the scTCR system. However, the levels of α chain secreted and associated with the β chain on the surface of the yeast (represented by the levels of c-Myc epitope) is

considerably lower, and it is unclear what fraction of this may have properly associated Vα and Vβ domains. Thus, the actual levels of TCRs displayed on the surface of the cells are likely to be lower than that seen in the scTCR system.

The second possibility, that the four-codon CDR3α libraries did not contain a solution that yielded higher affinity. It has been observed in the 2C system, that as many five contiguous mutations were likely necessary to isolate some of the high-affinity TCRs (94). In addition, it is possible that mutations in the CDR3β, or even other CDRs, may have generated TCR variants with higher affinity "solutions". These studies would require the generation of additional libraries to determine if they contained such high-affinity TCR variants.

Discussion

The ability to detect antigen-specific T cells is hindered by the low affinity of the TCR:pMHC interaction. However, Altman *et al* first demonstrated the ability to identify HIV specific $CD8^+$ T cells by adopting a strategy of tetramerizing biotinylated peptide-MHC complexes with fluorescently labeled streptavidin molecules (6). Since then, pMHC-tetramers have been invaluable in T cell biology research and also in clinical research including epitope discovery and mapping, vaccine and therapeutic development, and the analysis of disease responses (54). Multimerization of pMHC complexes have also since evolved with numerous formats being developed based on increasing the valency of interaction. Dimer pMHC complexes, using IgG as a molecular scaffold, were developed not long after pMHC tetramers, and have been commercialized by BD Biosciences as DimerX (116). Other groups adopted various

different scaffolds with which valency was increased that of tetramers. These include the pentameric pMHC (ProImmune), dextran conjugated dextramers (Immudex) and streptavidin-coated quantum dot conjugates, with the latter two reagents having between 20-30 pMHCs per conjugate (38, 176, 203). In many of these studies, it is likely that CD8 on the surface of the T cells also contributed to the binding of the multimeric pMHC (53). For purposes of in vitro engineering, for example by yeast display, there are additional problems with the use of these multimeric pMHC forms. CD8 is not available in the display systems, it is possible that TCR surface densities are below that found on T cells, and yeast cells do not have the membrane fluidity that allows improved multimeric detection. Accordingly, here I show the generation and application of a new multimeric reagent, based on streptavidin-coated fluorescent beads, that presents pMHCs at densities that are orders of magnitude higher than the aforementioned reagents, theoretically calculated to be ~$2x10^4$ per bead based on manufacturer's specifications.

Preceding the generation of libraries for affinity maturation, it is useful to verify that wild type receptors are properly folded on the surface of the yeast cell. In the absence of clonotypic antibodies such as those against conformational epitopes of the 2C TCR (108), or antibodies against conformational epitopes of the specific Vα and Vβ chains of the TCR of interest, the expression of functional TCRs on the surface of the yeast cell requires the probing of the specific TCR:pMHC interaction. However, while wild type TCRs with low affinity can be detected on the surface of T cells using available multimeric pMHCs reagents, they fail to do so when such receptors are displayed on the surface of yeast cells (43, 94, 95), presumably due to the lack of contribution from co-

receptor binding to pMHC, reduced densities, or minimal membrane fluidity. To address these issues, I demonstrated the ability to detect the low affinity TCR:pMHC interaction between yeast display wild-type 2C TCR and the SIY/K^b pMHC complex (K_D=30μM) using fluorescent pMHC beads. Thus, pMHC beads can be used as a probe for the expression of properly refolded of TCRs expressed in the yeast display system, as long as their affinities are above a threshold value of approximately 30 μM. While many wild-type TCRs are in this range, it is important to note that some TCRs have affinities with K_D values of 100 μM or even higher (32).

Dimeric and tetrameric pMHCs had been used for the isolation of high affinity mutants in the yeast display system of T cell engineering (94, 95), identifying mutations in CDR3α that bind to their cognate pMHC ligands with nanomolar affinities. In addition, it has been shown that mutations in CDR1 and CDR2 of both α and β chains of the TCR are also capable of enhancing binding affinities (47). Hence all six CDRs can be considered as potential targets for mutagenesis during library design for the engineering of high affinity TCRs. However, it is highly impractical to consider all CDRs as targets in a single library due to technical and practical considerations such as transformation efficiencies (10^7-10^8 transformants/μg plasmid in yeast display (14, 165)) and the speed of cell sorting without significant compromise to efficiency especially when collecting rare events (0.5-1% of cells sorted) (10). Thus, libraries design principles involve covering as many of the permutations as technically possible. In libraries where a higher affinity “solution” is below the sensitivity of reagents used during the screening process, it would not be selected. However, improved sensitivity with the pMHC beads would allow the isolation of such intermediate affinity mutants. These mutants can often be

used as leads upon which new libraries can be made for further enhancement. In addition, rational design of libraries that incorporates mutations identified in similar leads from individual libraries could be done to generate improved affinity mutants that would had been impossible from a single library (217). This strategy may be most useful when attempting to isolate *de novo* binders from naïve libraries.

Lastly, it is worth noting that there are applications in which the highest affinity solutions are not the most ideal target. In the application of soluble TCRs as a targeting tool, high affinity receptors are likely necessary, as stronger binding facilitate the detection of target cells and increase the efficacy of therapeutic payloads. In addition, as specific pMHC ligands on the surface of target cells are found in low numbers the use of multimeric TCR complexes would be impractical due to the lack of avidity. However, another possible application for affinity engineered TCRs is TCR gene therapy, where a patients' T cells are transduced to express enhanced TCRs and reintroduced into the body where the modified T cells would elicit improved responses against virally infected or tumor cells (178). However, recent studies had cautioned against the use of high affinity receptors in such gene therapies. Zhao *et al.* showed that in $CD8^+$ T cells expressing high affinity receptors lost antigen specificity while receptors with intermediate affinities (0.5 - 4μM) were able to maintain their specificity (166, 228). It has also been shown in our lab that cells expressing the high affinity m33 TCR showed cross-reactivity (94). Thomas *et al.* also demonstrated that while T cells expressing higher affinity receptors exhibited faster responses, their sensitivity to recognize antigens at low density was also affected (196). The 'productive hit rate' model states that serial engagement of TCRs is required for the amplification of

intracellular signals for T cell activation (206). In line with this model, Kalergis *et al*. and Dushek *et al.* proposed an explanation for the above observation by suggesting that an optimal k_{off} or dwell time exists for efficient T cell activation (62, 107), beyond which the efficiency of serial engagement would be reduced and thus impaired T cell activation. Since T cell receptors with intermediate affinities were shown to confer co-receptor independence, allowing $CD8^-$ and CD4+ cells to respond to antigen stimulation (43, 166, 228), the use of TCRs with intermediate affinities may be more ideal in TCR gene therapy.

In summary, pMHC beads for sorting of TCR libraries provides a screening strategy would allow us to isolate a broader range of TCRs with higher affinity, useful as intermediate leads in further engineering for soluble TCRs applications, and also providing intermediate affinity TCRs for use in adoptive T cell therapies with transduced TCRs.

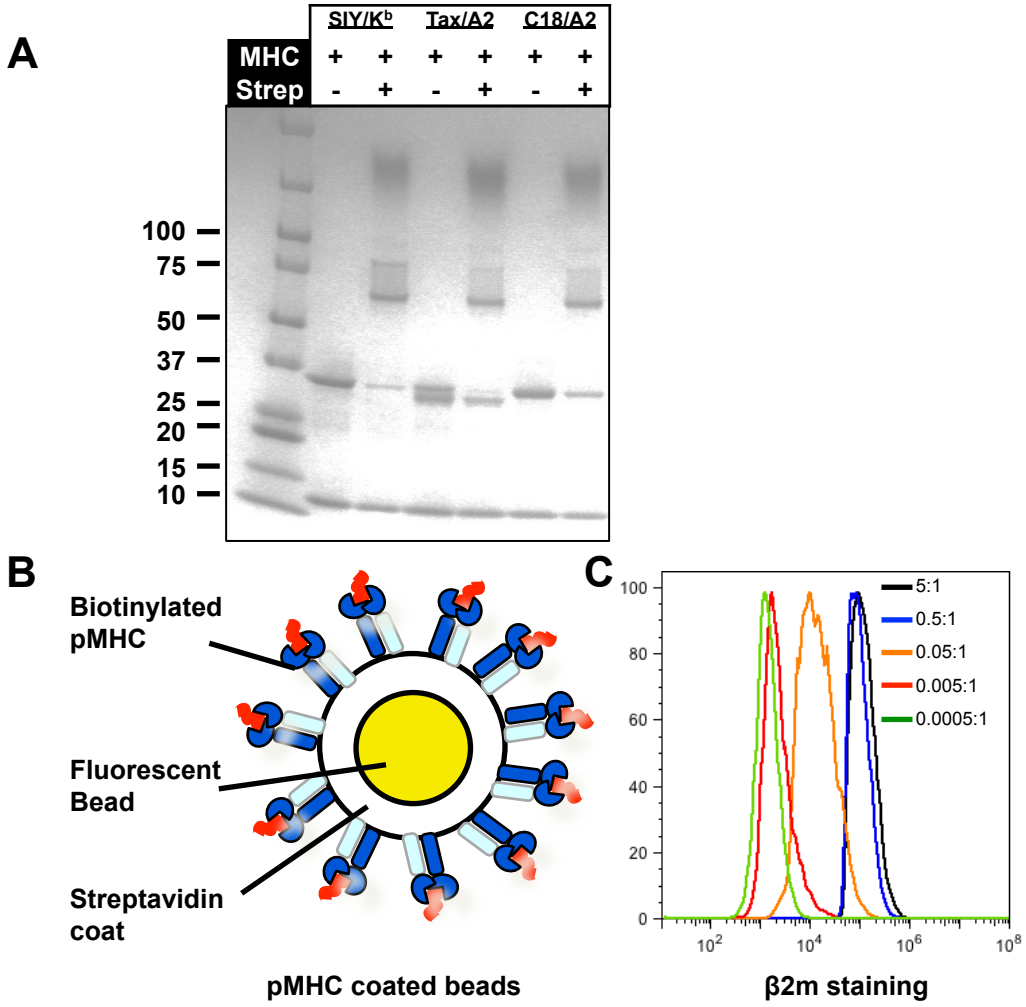


Figure 4.1. Production of pMHC conjugated beads. Inclusion bodies of K^b and HLA-A*02:01 heavy chains and β2m were refolded with excess of peptides and purified. (A) Biotinylation was done in vitro by enzymatic conjugation with biotin ligase. Level of biotinylation was then probed by a gel shift assay done using SDS-PAGE in the presence and absence of excess streptavidin. (B) Schematic of pMHC coated fluorescent beads. (C) Level of pMHC conjugation can be probed by staining with an anti-β2m antibody. Beads were loaded with a titration of pMHC concentrations, from 5:1 to 0.0005:1 pMHC to biotin binding site ratio, generating beads with various density of pMHCs.

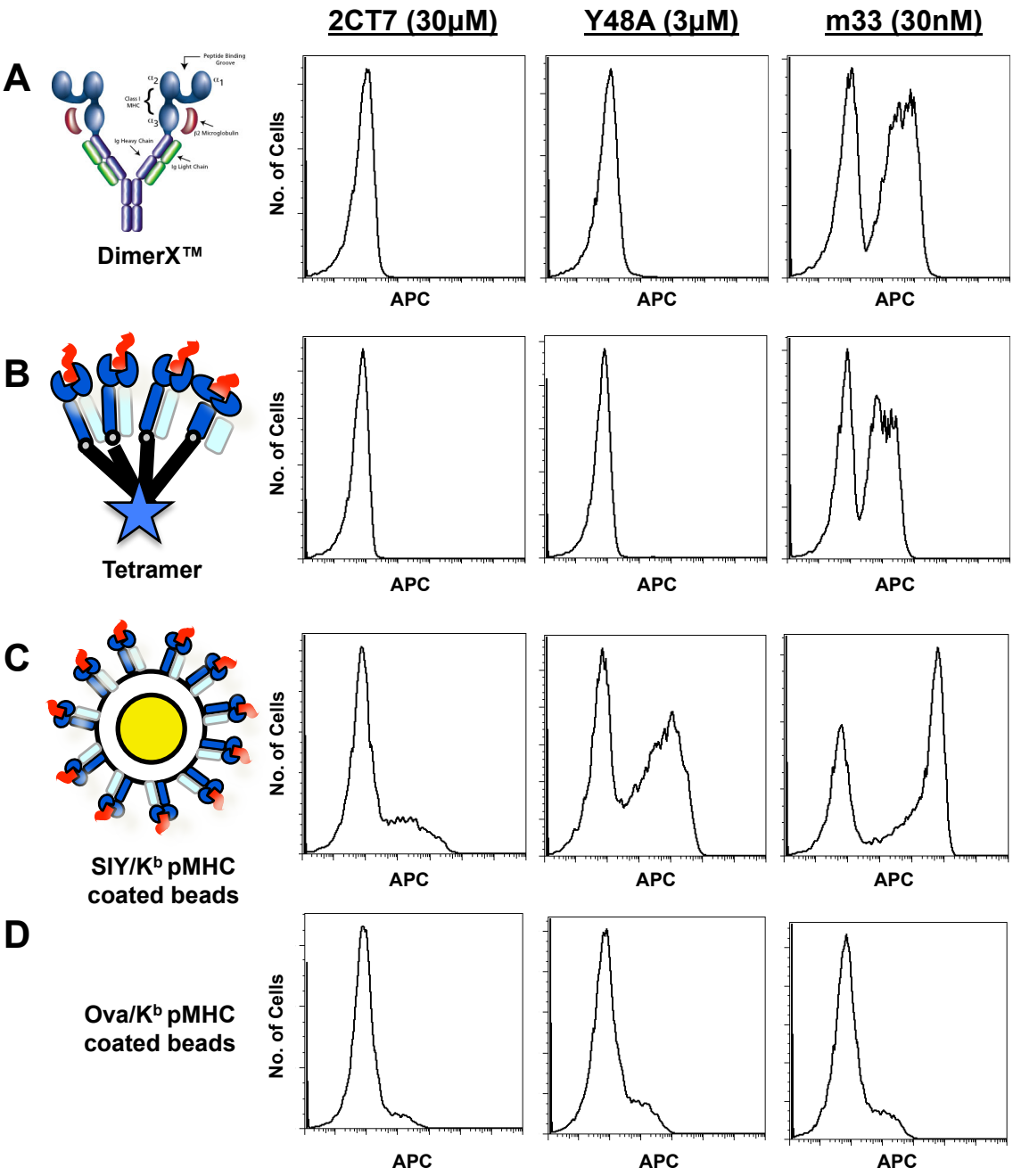


Figure 4.2. Detection of yeast displayed single chain T cell receptors with multimeric pMHC detection reagents. 2CT7 scTCR with wild-type affinity of 30μM along with the higher affinity variants Y48A (3μM) and m33 (30nM) were stained with equal concentrations of SIY/K^b presented in three different forms of conjugations. Comparison shows that at the same concentration of pMHC used, the coated beads was the only one that was able to detectably bind all three TCRs displayed on yeast cells.

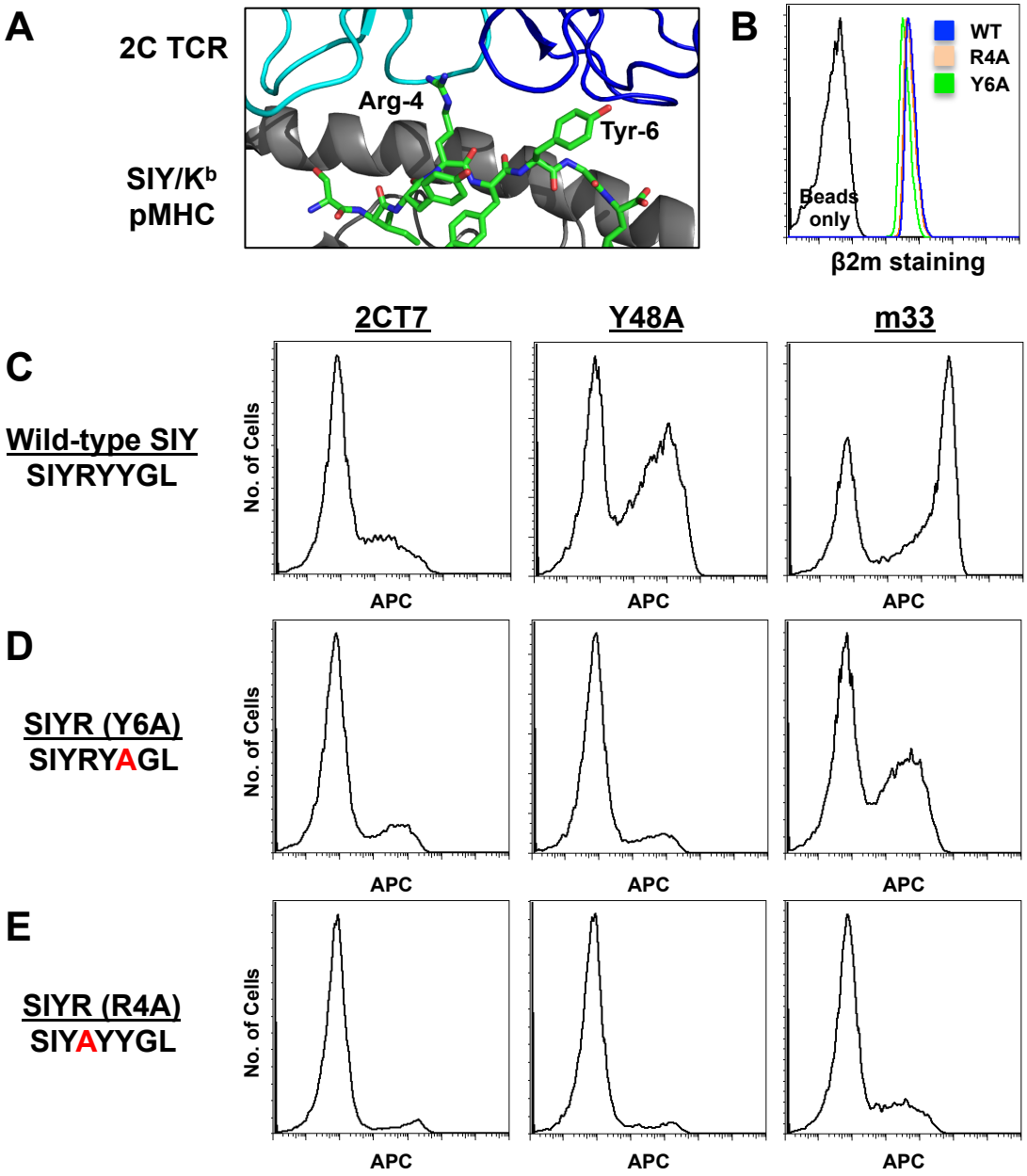


Figure 4.3. Staining of yeast displayed 2C TCR and higher affinity mutants with pMHC beads presenting SIY peptide variants. (A) Structure of the 2C-SIY/K^b complex (PDB code: 1G6R) highlighting the protrusion of the side chains of Arg at position 4 and Tyr at position 6 of the SIY peptide toward the TCR (α chain in cyan, β chain in blue). (B) Refolded recombinant K^b in presenting wild-type SIY, R4A and Y6A variants were conjugated onto streptavidin coated beads to saturation. 2CT7, Y48A and m33 were stained with equal concentrations of SIY K^b presenting wild type and single Ala substituted peptide variants Y6A (D) and R4A (E).

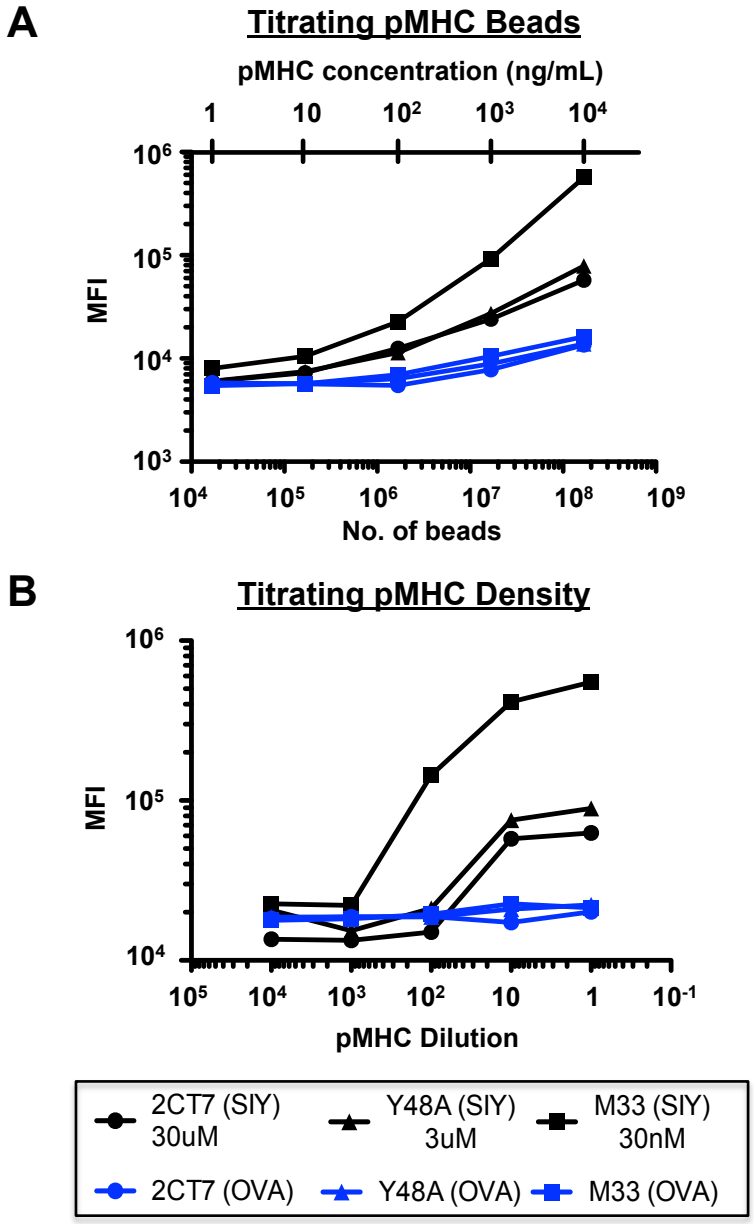


Figure 4.4. Sensitivity of pMHC beads. (A) Yeast cells expressing 2CT7, Y48A and m33 cells were stained with varying numbers of beads saturated with pMHC. Theoretical pMHC concentrations presented by the beads were calculated based on manufacturer's specifications. (B) Density of pMHC presented by beads were varied by titrating the concentration of biotinylated pMHC used for conjugation, starting with 5 fold excess, and subsequently used to stain the yeast cells.

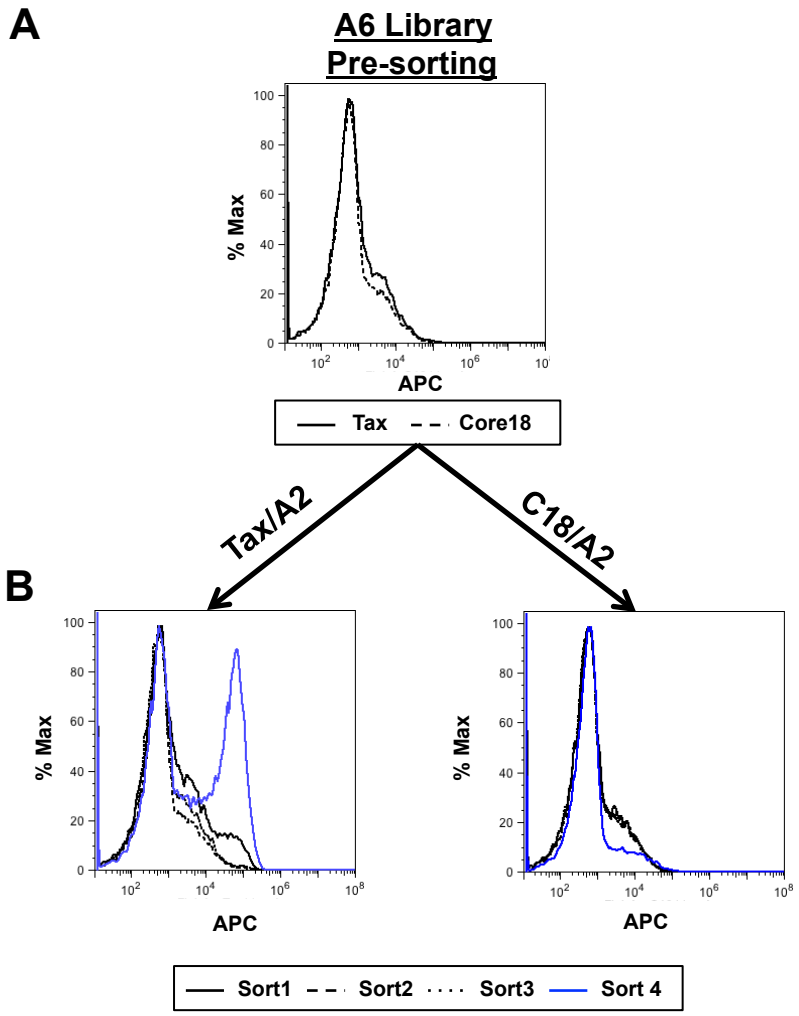


Figure 4.5. Sorting an A6 scTCR library with Tax/A2 beads. (A) The A6 scaffold library was stained with beads saturated with Tax/A2 and C18/A2 pMHCs. (B) 10^8 cells were sorted with the top 1% of binders were collected during the first two rounds and top 0.5% of binders collected in the 3rd and 4th rounds. After 4 sorts, yeast cells that were binding Tax/A2 beads were enriched but not for Core18/A2 beads.

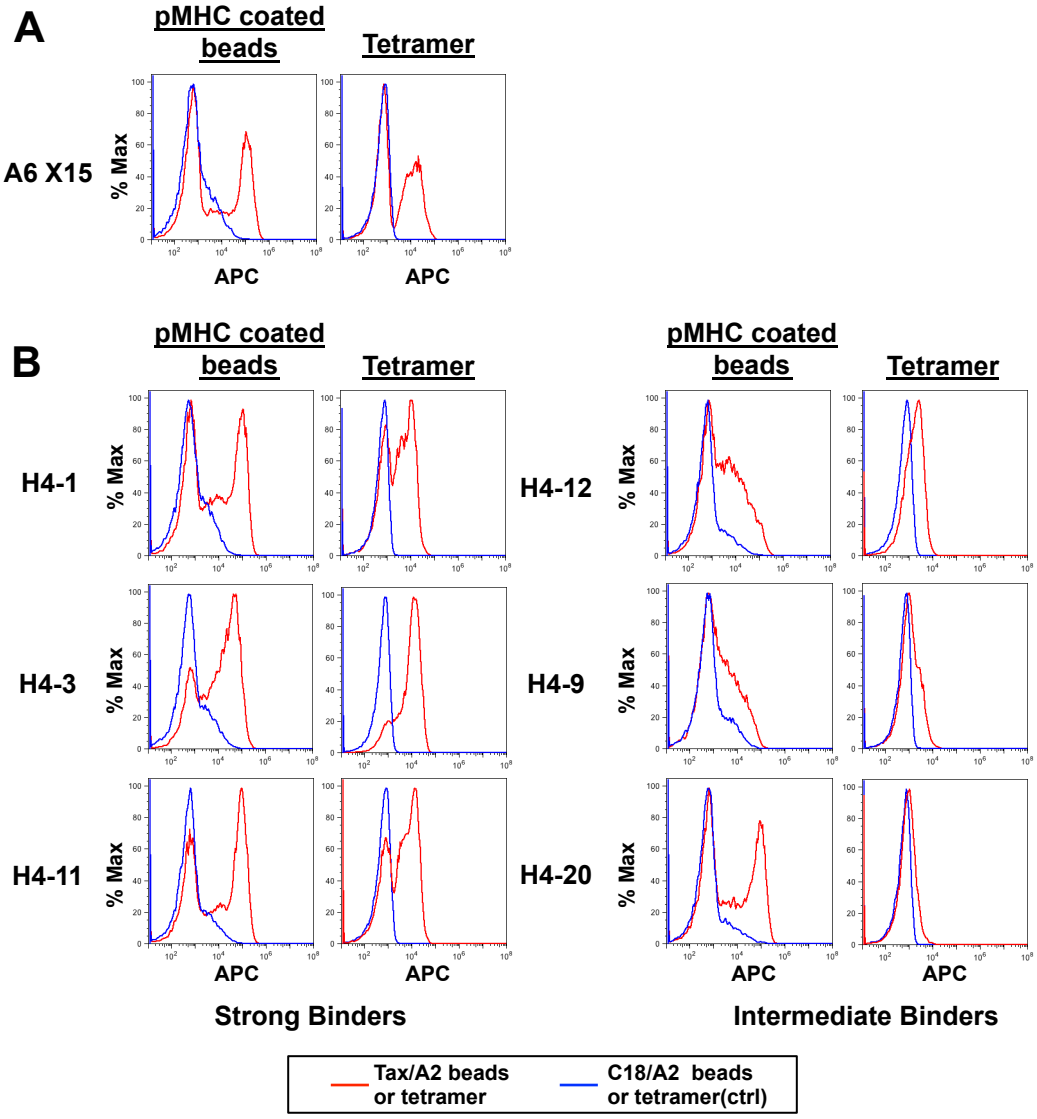


Figure 4.6. Analysis of clones isolated from A6 scTCR library using Tax/A2 pMHC beads. 20 clones were picked from individual colonies isolated from the cells collected enriched from the A6 scaffold library for binding to Tax/A2 pMHC coated beads. Strong binders with binding profiles similar to the original A6 X15 clone were observed in at least 50% of the clones isolated while 20% of the clones were found to be able to bind pMHC coated beads moderately well but poorly to tetramers.

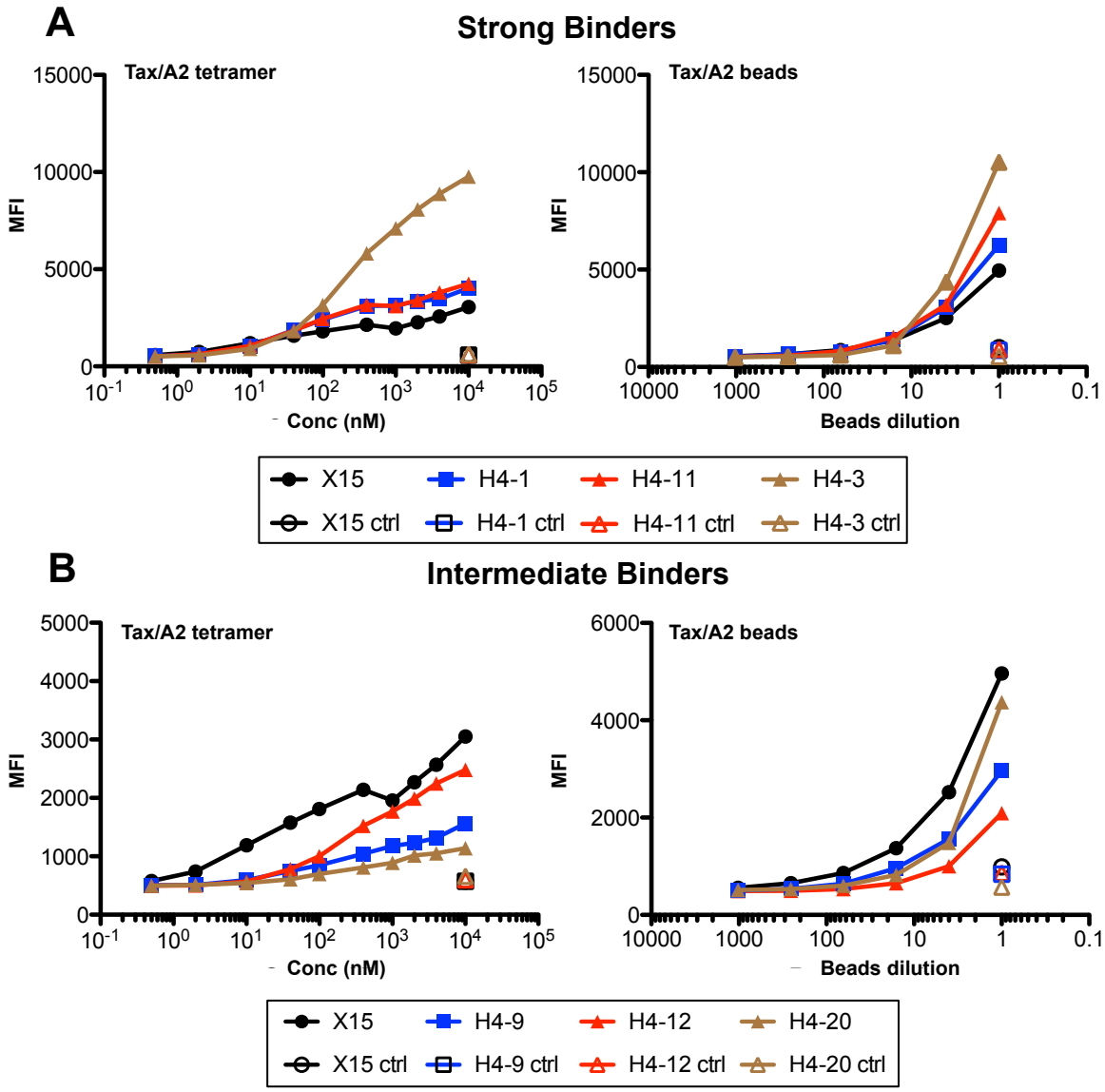


Figure 4.7. Beads and tetramer titration analysis of 3 strong and 3 intermediate binding clones. Clones H4-1, H4-3 and H4-11 (A) and clones H4-9, H4-12 and H4-20 (B) were stained with a titration of Tax/A2 pMHC tetramers (left panel) and Tax/A2 pMHC coated beads (right panel). Clones H4-1, H4-3 and H4-11 showed similar binding affinity as the high affinity A6 X15 clone (black). C18/A2 pMHC coated beads were used as controls (ctrl).

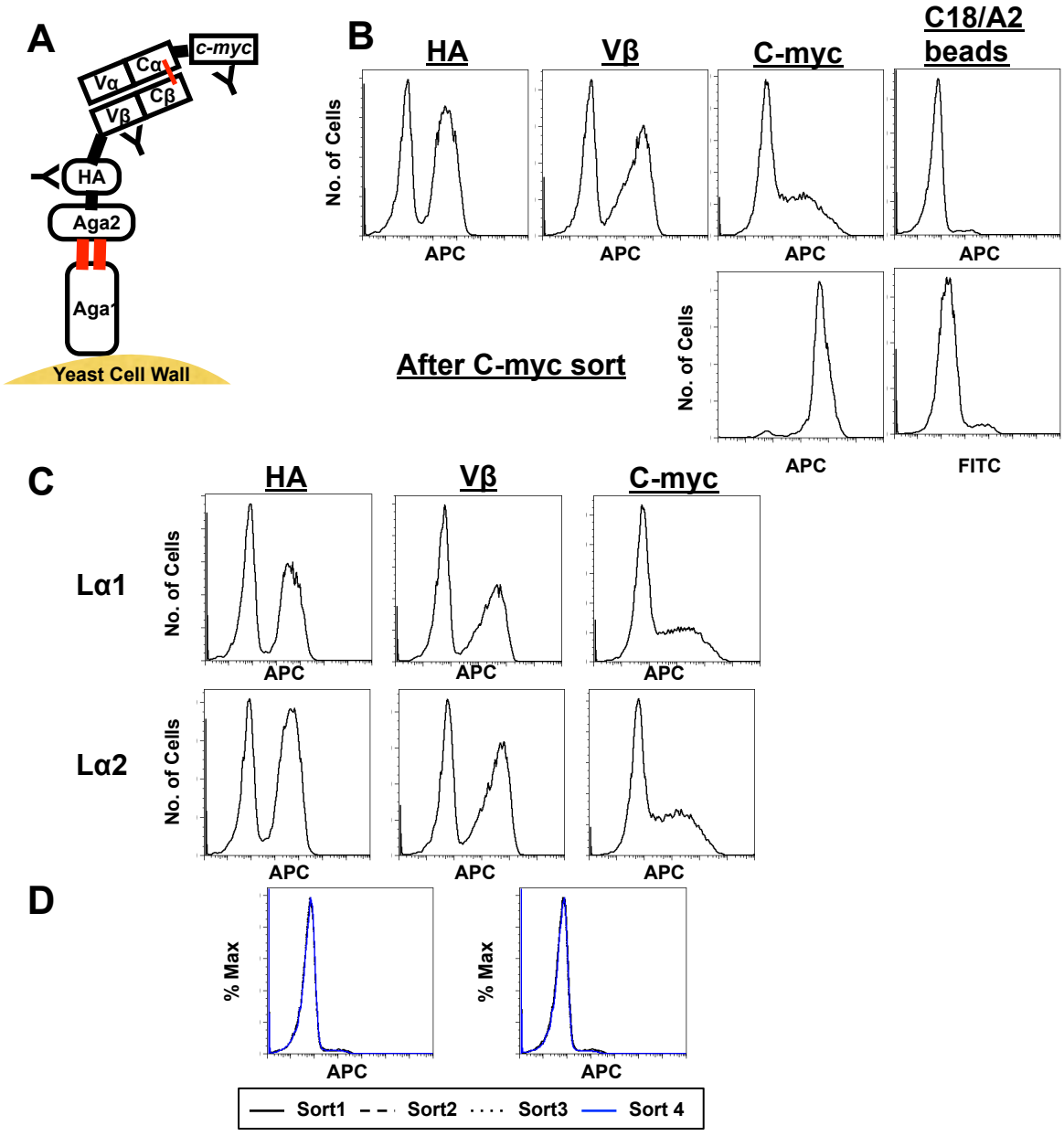


Figure 4.8. Sorting a full length C18/A2 TCR library sorting with C18/A2 beads . (A) Full length wild type C18/A2 specific TCR was expressed on the surface of yeast cells with the β chain tethered to the AGA complex and the α chain secreted. Association of the 2 chains was facilitated by an engineered disulfide bond (red line). (B) Expression was probed by antibodies against c-myc (α chain), Vβ and HA (β chain). Yeast cells sorted for c-myc expression and immediately stained with C18/A2 beads did not show improved staining. (B) Expression of α and β chains in 2 full length CDR3α libraries, Lα1 and Lα2. (C) Lα1 and Lα2 were subjected to 4 rounds of sorting, isolating the top 1% binders for the first 2 sorts and the top 0.5% binders in the 3rd and 4th sorts. No enrichment was observed for both libraries after 4 sorts.

Library	Sequence
Wild type	LSGSARQ
Lα1	XXXXARQ
Lα2	LSGXXXX

Table 4.1. HBV Core $_{18\text{-}27}$ specific TCR CDR3α library design. Two libraries were made with 4 amino acids randomized (X) at positions covering the length CDR3α sequences.

REFERENCES

1. **Abbas, A. K., K. M. Murphy, and A. Sher.** 1996. Functional diversity of helper T lymphocytes. Nature **383:**787-793.
2. **Aggen, D. H., A. S. Chervin, F. K. Insaidoo, K. H. Piepenbrink, B. M. Baker, and D. M. Kranz.** 2011. Identification and engineering of human variable regions that allow expression of stable single-chain T cell receptors. Protein engineering, design & selection : PEDS **24:**361-372.
3. **Agrawal, A., Q. M. Eastman, and D. G. Schatz.** 1998. Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. Nature **394:**744-751.
4. **Agrawal, A., A. K. Shrive, T. J. Greenhough, and J. E. Volanakis.** 2001. Topology and structure of the C1q-binding site on C-reactive protein. J Immunol **166:**3998-4004.
5. **Alarcon, B., M. Swamy, H. M. van Santen, and W. W. Schamel.** 2006. T-cell antigen-receptor stoichiometry: pre-clustering for sensitivity. EMBO reports **7:**490-495.
6. **Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis.** 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science **274:**94-96.
7. **Anderson, M. W., and J. Gorski.** 2003. Cutting edge: TCR contacts as anchors: effects on affinity and HLA-DM stability. J Immunol **171:**5683-5687.
8. **Anikeeva, N., T. Lebedeva, M. Sumaroka, S. A. Kalams, and Y. Sykulev.** 2003. Soluble HIV-specific T cell receptor: expression, purification and analysis of the specificity. Journal of immunological methods **277:**75-86.
9. **Anikeeva, N., T. Mareeva, W. Liu, and Y. Sykulev.** 2009. Can oligomeric T-cell receptor be used as a tool to detect viral peptide epitopes on infected cells? Clin Immunol **130:**98-109.
10. **Arnold, L. W., and J. Lannigan.** 2010. Practical issues in high-speed cell sorting. Current protocols in cytometry / editorial board, J. Paul Robinson, managing editor ... [et al.] **Chapter 1:**Unit 1 24 21-30.
11. **Bakker, A. H., R. Hoppes, C. Linnemann, M. Toebes, B. Rodenko, C. R. Berkers, S. R. Hadrup, W. J. van Esch, M. H. Heemskerk, H. Ovaa, and T. N. Schumacher.** 2008. Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7. Proceedings of the National Academy of Sciences of the United States of America **105:**3825-3830.
12. **Bankovich, A. J., and K. C. Garcia.** 2003. Not just any T cell receptor will do. Immunity **18:**7-11.
13. **Bargou, R., E. Leo, G. Zugmaier, M. Klinger, M. Goebeler, S. Knop, R. Noppeney, A. Viardot, G. Hess, M. Schuler, H. Einsele, C. Brandl, A. Wolf, P. Kirchinger, P. Klappers, M. Schmidt, G. Riethmuller, C. Reinhardt, P. A. Baeuerle, and P. Kufer.** 2008. Tumor regression in cancer patients by very low doses of a T cell-engaging antibody. Science **321:**974-977.

14. **Benatuil, L., J. M. Perez, J. Belk, and C. M. Hsieh.** 2010. An improved yeast transformation method for the generation of very large human antibody libraries. Protein engineering, design & selection : PEDS **23:**155-159.
15. **Bentley, G. A., and R. A. Mariuzza.** 1996. The structure of the T cell antigen receptor. Annual review of immunology **14:**563-590.
16. **Berthillon, P., J. M. Crance, F. Leveque, A. Jouan, M. A. Petit, R. Deloince, and C. Trepo.** 1996. Inhibition of the expression of hepatitis A and B viruses (HAV and HBV) proteins by interferon in a human hepatocarcinoma cell line (PLC/PRF/5). Journal of hepatology **25:**15-19.
17. **Bertoletti, A., F. V. Chisari, A. Penna, S. Guilhot, L. Galati, G. Missale, P. Fowler, H. J. Schlicht, A. Vitiello, R. C. Chesnut, and et al.** 1993. Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. Journal of virology **67:**2376-2380.
18. **Bertoletti, A., A. Costanzo, F. V. Chisari, M. Levrero, M. Artini, A. Sette, A. Penna, T. Giuberti, F. Fiaccadori, and C. Ferrari.** 1994. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. The Journal of experimental medicine **180:**933-943.
19. **Bertoletti, A., C. Ferrari, F. Fiaccadori, A. Penna, R. Margolskee, H. J. Schlicht, P. Fowler, S. Guilhot, and F. V. Chisari.** 1991. HLA class I-restricted human cytotoxic T cells recognize endogenously synthesized hepatitis B virus nucleocapsid antigen. Proceedings of the National Academy of Sciences of the United States of America **88:**10445-10449.
20. **Bertoletti, A., and A. J. Gehring.** 2006. The immune response during hepatitis B virus infection. The Journal of general virology **87:**1439-1449.
21. **Bertoletti, A., and M. K. Maini.** 2000. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? Current opinion in immunology **12:**403-408.
22. **Bertoletti, A., A. Sette, F. V. Chisari, A. Penna, M. Levrero, M. De Carli, F. Fiaccadori, and C. Ferrari.** 1994. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. Nature **369:**407-410.
23. **Biddison, W. E., R. V. Turner, S. J. Gagnon, A. Lev, C. J. Cohen, and Y. Reiter.** 2003. Tax and M1 peptide/HLA-A2-specific Fabs and T cell receptors recognize nonidentical structural features on peptide/HLA-A2 complexes. J Immunol **171:**3064-3074.
24. **Blasius, A. L., and B. Beutler.** 2010. Intracellular toll-like receptors. Immunity **32:**305-315.
25. **Boder, E. T., and K. D. Wittrup.** 1997. Yeast surface display for screening combinatorial polypeptide libraries. Nature biotechnology **15:**553-557.
26. **Boehm, U., T. Klamp, M. Groot, and J. C. Howard.** 1997. Cellular responses to interferon-gamma. Annual review of immunology **15:**749-795.
27. **Bogdan, C., M. Rollinghoff, and A. Diefenbach.** 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Current opinion in immunology **12:**64-76.
28. **Boon, T., and P. van der Bruggen.** 1996. Human tumor antigens recognized by T lymphocytes. The Journal of experimental medicine **183:**725-729.

29. **Boublik, Y., P. Di Bonito, and I. M. Jones.** 1995. Eukaryotic virus display: engineering the major surface glycoprotein of the Autographa californica nuclear polyhedrosis virus (AcNPV) for the presentation of foreign proteins on the virus surface. Biotechnology (N Y) **13:**1079-1084.
30. **Boulter, J. M., M. Glick, P. T. Todorov, E. Baston, M. Sami, P. Rizkallah, and B. K. Jakobsen.** 2003. Stable, soluble T-cell receptor molecules for crystallization and therapeutics. Protein engineering **16:**707-711.
31. **Bowerman, N. A., L. A. Colf, K. C. Garcia, and D. M. Kranz.** 2009. Different strategies adopted by K(b) and L(d) to generate T cell specificity directed against their respective bound peptides. The Journal of biological chemistry **284:**32551-32561.
32. **Bowerman, N. A., T. S. Crofts, L. Chlewicki, P. Do, B. M. Baker, K. Christopher Garcia, and D. M. Kranz.** 2009. Engineering the binding properties of the T cell receptor:peptide:MHC ternary complex that governs T cell activity. Molecular immunology **46:**3000-3008.
33. **Bowley, D. R., A. F. Labrijn, M. B. Zwick, and D. R. Burton.** 2007. Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. Protein engineering, design & selection : PEDS **20:**81-90.
34. **Buonpane, R. A., H. R. Churchill, B. Moza, E. J. Sundberg, M. L. Peterson, P. M. Schlievert, and D. M. Kranz.** 2007. Neutralization of staphylococcal enterotoxin B by soluble, high-affinity receptor antagonists. Nature medicine **13:**725-729.
35. **Card, K. F., S. A. Price-Schiavi, B. Liu, E. Thomson, E. Nieves, H. Belmont, J. Builes, J. A. Jiao, J. Hernandez, J. Weidanz, L. Sherman, J. L. Francis, A. Amirkhosravi, and H. C. Wong.** 2004. A soluble single-chain T-cell receptor IL-2 fusion protein retains MHC-restricted peptide specificity and IL-2 bioactivity. Cancer immunology, immunotherapy : CII **53:**345-357.
36. **Cariappa, A., and S. Pillai.** 2002. Antigen-dependent B-cell development. Current opinion in immunology **14:**241-249.
37. **Chamoto, K., T. Tsuji, H. Funamoto, A. Kosaka, J. Matsuzaki, T. Sato, H. Abe, K. Fujio, K. Yamamoto, T. Kitamura, T. Takeshima, Y. Togashi, and T. Nishimura.** 2004. Potentiation of tumor eradication by adoptive immunotherapy with T-cell receptor gene-transduced T-helper type 1 cells. Cancer research **64:**386-390.
38. **Chattopadhyay, P. K., D. A. Price, T. F. Harper, M. R. Betts, J. Yu, E. Gostick, S. P. Perfetto, P. Goepfert, R. A. Koup, S. C. De Rosa, M. P. Bruchez, and M. Roederer.** 2006. Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry. Nature medicine **12:**972-977.
39. **Chen, H., Z. M. Ndhlovu, D. Liu, L. C. Porter, J. W. Fang, S. Darko, M. A. Brockman, T. Miura, Z. L. Brumme, A. Schneidewind, A. Piechocka-Trocha, K. T. Cesa, J. Sela, T. D. Cung, I. Toth, F. Pereyra, X. G. Yu, D. C. Douek, D. E. Kaufmann, T. M. Allen, and B. D. Walker.** 2012. TCR clonotypes modulate the protective effect of HLA class I molecules in HIV-1 infection. Nature immunology **13:**691-700.

40. **Chen, M. T., J. N. Billaud, M. Sallberg, L. G. Guidotti, F. V. Chisari, J. Jones, J. Hughes, and D. R. Milich.** 2004. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. Proceedings of the National Academy of Sciences of the United States of America **101:**14913-14918.
41. **Chervin, A. S., D. H. Aggen, J. M. Raseman, and D. M. Kranz.** 2008. Engineering higher affinity T cell receptors using a T cell display system. Journal of immunological methods **339:**175-184.
42. **Chervin, A. S., J. D. Stone, N. A. Bowerman, and D. M. Kranz.** 2009. Cutting edge: inhibitory effects of CD4 and CD8 on T cell activation induced by high-affinity noncognate ligands. J Immunol **183:**7639-7643.
43. **Chervin, A. S., J. D. Stone, P. D. Holler, A. Bai, J. Chen, H. N. Eisen, and D. M. Kranz.** 2009. The impact of TCR-binding properties and antigen presentation format on T cell responsiveness. J Immunol **183:**1166-1178.
44. **Chew, S. L., M. Y. Or, C. X. Chang, A. J. Gehring, A. Bertoletti, and G. M. Grotenbreg.** 2011. Stability screening of arrays of major histocompatibility complexes on combinatorially encoded flow cytometry beads. The Journal of biological chemistry **286:**28466-28475.
45. **Chicz, R. M., R. G. Urban, W. S. Lane, J. C. Gorga, L. J. Stern, D. A. Vignali, and J. L. Strominger.** 1992. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature **358:**764-768.
46. **Chisari, F. V., and C. Ferrari.** 1995. Hepatitis B virus immunopathogenesis. Annual review of immunology **13:**29-60.
47. **Chlewicki, L. K., P. D. Holler, B. C. Monti, M. R. Clutter, and D. M. Kranz.** 2005. High-affinity, peptide-specific T cell receptors can be generated by mutations in CDR1, CDR2 or CDR3. Journal of molecular biology **346:**223-239.
48. **Clay, T. M., M. C. Custer, J. Sachs, P. Hwu, S. A. Rosenberg, and M. I. Nishimura.** 1999. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. J Immunol **163:**507-513.
49. **Cohen, C. J., O. Sarig, Y. Yamano, U. Tomaru, S. Jacobson, and Y. Reiter.** 2003. Direct phenotypic analysis of human MHC class I antigen presentation: visualization, quantitation, and in situ detection of human viral epitopes using peptide-specific, MHC-restricted human recombinant antibodies. J Immunol **170:**4349-4361.
50. **Cole, D. K., N. J. Pumphrey, J. M. Boulter, M. Sami, J. I. Bell, E. Gostick, D. A. Price, G. F. Gao, A. K. Sewell, and B. K. Jakobsen.** 2007. Human TCR-binding affinity is governed by MHC class restriction. J Immunol **178:**5727-5734.
51. **Cooper, L. J., M. Kalos, D. A. Lewinsohn, S. R. Riddell, and P. D. Greenberg.** 2000. Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes. Journal of virology **74:**8207-8212.
52. **Cooper, M. D., and M. N. Alder.** 2006. The evolution of adaptive immune systems. Cell **124:**815-822.

53. **Daniels, M. A., and S. C. Jameson.** 2000. Critical role for CD8 in T cell receptor binding and activation by peptide/major histocompatibility complex multimers. The Journal of experimental medicine **191:**335-346.
54. **Davis, M. M., J. D. Altman, and E. W. Newell.** 2011. Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. Nature reviews. Immunology **11:**551-558.
55. **Davis, M. M., J. J. Boniface, Z. Reich, D. Lyons, J. Hampl, B. Arden, and Y. Chien.** 1998. Ligand recognition by alpha beta T cell receptors. Annual review of immunology **16:**523-544.
56. **Deng, L., R. J. Langley, P. H. Brown, G. Xu, L. Teng, Q. Wang, M. I. Gonzales, G. G. Callender, M. I. Nishimura, S. L. Topalian, and R. A. Mariuzza.** 2007. Structural basis for the recognition of mutant self by a tumor-specific, MHC class II-restricted T cell receptor. Nature immunology **8:**398-408.
57. **Denkberg, G., C. J. Cohen, A. Lev, P. Chames, H. R. Hoogenboom, and Y. Reiter.** 2002. Direct visualization of distinct T cell epitopes derived from a melanoma tumor-associated antigen by using human recombinant antibodies with MHC- restricted T cell receptor-like specificity. Proceedings of the National Academy of Sciences of the United States of America **99:**9421-9426.
58. **Dhawan, A., J. Puppi, R. D. Hughes, and R. R. Mitry.** 2010. Human hepatocyte transplantation: current experience and future challenges. Nature reviews. Gastroenterology & hepatology **7:**288-298.
59. **Donermeyer, D. L., K. S. Weber, D. M. Kranz, and P. M. Allen.** 2006. The study of high-affinity TCRs reveals duality in T cell recognition of antigen: specificity and degeneracy. J Immunol **177:**6911-6919.
60. **Dunkelberger, J. R., and W. C. Song.** 2010. Complement and its role in innate and adaptive immune responses. Cell research **20:**34-50.
61. **Dunn, S. M., P. J. Rizkallah, E. Baston, T. Mahon, B. Cameron, R. Moysey, F. Gao, M. Sami, J. Boulter, Y. Li, and B. K. Jakobsen.** 2006. Directed evolution of human T cell receptor CDR2 residues by phage display dramatically enhances affinity for cognate peptide-MHC without increasing apparent cross-reactivity. Protein science : a publication of the Protein Society **15:**710-721.
62. **Dushek, O., M. Aleksic, R. J. Wheeler, H. Zhang, S. P. Cordoba, Y. C. Peng, J. L. Chen, V. Cerundolo, T. Dong, D. Coombs, and P. A. van der Merwe.** 2011. Antigen potency and maximal efficacy reveal a mechanism of efficient T cell activation. Science signaling **4:**ra39.
63. **Epel, M., J. D. Ellenhorn, D. J. Diamond, and Y. Reiter.** 2002. A functional recombinant single-chain T cell receptor fragment capable of selectively targeting antigen-presenting cells. Cancer immunology, immunotherapy : CII **51:**565-573.
64. **Falk, K., O. Rotzschke, K. Deres, J. Metzger, G. Jung, and H. G. Rammensee.** 1991. Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. The Journal of experimental medicine **174:**425-434.
65. **Falk, K., O. Rotzschke, S. Stevanovic, G. Jung, and H. G. Rammensee.** 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature **351:**290-296.

66. **Feldhaus, M. J., R. W. Siegel, L. K. Opresko, J. R. Coleman, J. M. Feldhaus, Y. A. Yeung, J. R. Cochran, P. Heinzelman, D. Colby, J. Swers, C. Graff, H. S. Wiley, and K. D. Wittrup.** 2003. Flow-cytometric isolation of human antibodies from a nonimmune Saccharomyces cerevisiae surface display library. Nature biotechnology **21:**163-170.
67. **Ferrari, C., A. Bertoletti, A. Penna, A. Cavalli, A. Valli, G. Missale, M. Pilli, P. Fowler, T. Giuberti, F. V. Chisari, and et al.** 1991. Identification of immunodominant T cell epitopes of the hepatitis B virus nucleocapsid antigen. The Journal of clinical investigation **88:**214-222.
68. **Ferrari, C., G. Missale, C. Boni, and S. Urbani.** 2003. Immunopathogenesis of hepatitis B. Journal of hepatology **39 Suppl 1:**S36-42.
69. **Ferrari, C., A. Penna, A. Bertoletti, A. Valli, A. D. Antoni, T. Giuberti, A. Cavalli, M. A. Petit, and F. Fiaccadori.** 1990. Cellular immune response to hepatitis B virus-encoded antigens in acute and chronic hepatitis B virus infection. J Immunol **145:**3442-3449.
70. **Fooksman, D. R., G. K. Gronvall, Q. Tang, and M. Edidin.** 2006. Clustering class I MHC modulates sensitivity of T cell recognition. J Immunol **176:**6673-6680.
71. **Foote, J., and H. N. Eisen.** 2000. Breaking the affinity ceiling for antibodies and T cell receptors. Proceedings of the National Academy of Sciences of the United States of America **97:**10679-10681.
72. **Fraser, I. P., H. Koziel, and R. A. Ezekowitz.** 1998. The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. Seminars in immunology **10:**363-372.
73. **Frickel, E. M., N. Sahoo, J. Hopp, M. J. Gubbels, M. P. Craver, L. J. Knoll, H. L. Ploegh, and G. M. Grotenbreg.** 2008. Parasite stage-specific recognition of endogenous Toxoplasma gondii-derived CD8+ T cell epitopes. The Journal of infectious diseases **198:**1625-1633.
74. **Ganz, T.** 2003. Defensins: antimicrobial peptides of innate immunity. Nature reviews. Immunology **3:**710-720.
75. **Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. E. Biddison, and D. C. Wiley.** 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. Nature **384:**134-141.
76. **Garcia, K. C., and E. J. Adams.** 2005. How the T cell receptor sees antigen--a structural view. Cell **122:**333-336.
77. **Garcia, K. C., M. Degano, R. L. Stanfield, A. Brunmark, M. R. Jackson, P. A. Peterson, L. Teyton, and I. A. Wilson.** 1996. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. Science **274:**209-219.
78. **Garcia, K. C., M. D. Tallquist, L. R. Pease, A. Brunmark, C. A. Scott, M. Degano, E. A. Stura, P. A. Peterson, I. A. Wilson, and L. Teyton.** 1997. Alphabeta T cell receptor interactions with syngeneic and allogeneic ligands: affinity measurements and crystallization. Proceedings of the National Academy of Sciences of the United States of America **94:**13838-13843.

79. **Gehring, A. J., D. Sun, P. T. Kennedy, E. Nolte-'t Hoen, S. G. Lim, S. Wasser, C. Selden, M. K. Maini, D. M. Davis, M. Nassal, and A. Bertoletti.** 2007. The level of viral antigen presented by hepatocytes influences CD8 T-cell function. Journal of virology **81:**2940-2949.
80. **Gehring, A. J., S. A. Xue, Z. Z. Ho, D. Teoh, C. Ruedl, A. Chia, S. Koh, S. G. Lim, M. K. Maini, H. Stauss, and A. Bertoletti.** 2011. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. Journal of hepatology **55:**103-110.
81. **Germain, R. N., and D. H. Margulies.** 1993. The biochemistry and cell biology of antigen processing and presentation. Annual review of immunology **11:**403-450.
82. **Ghosh, S., M. J. May, and E. B. Kopp.** 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annual review of immunology **16:**225-260.
83. **Gredmark-Russ, S., E. J. Cheung, M. K. Isaacson, H. L. Ploegh, and G. M. Grotenbreg.** 2008. The CD8 T-cell response against murine gammaherpesvirus 68 is directed toward a broad repertoire of epitopes from both early and late antigens. Journal of virology **82:**12205-12212.
84. **Greenberg, S., and S. Grinstein.** 2002. Phagocytosis and innate immunity. Current opinion in immunology **14:**136-145.
85. **Grotenbreg, G. M., N. R. Roan, E. Guillen, R. Meijers, J. H. Wang, G. W. Bell, M. N. Starnbach, and H. L. Ploegh.** 2008. Discovery of CD8+ T cell epitopes in Chlamydia trachomatis infection through use of caged class I MHC tetramers. Proceedings of the National Academy of Sciences of the United States of America **105:**3831-3836.
86. **Guidotti, L. G., and F. V. Chisari.** 2006. Immunobiology and pathogenesis of viral hepatitis. Annual review of pathology **1:**23-61.
87. **Guidotti, L. G., S. Guilhot, and F. V. Chisari.** 1994. Interleukin-2 and alpha/beta interferon down-regulate hepatitis B virus gene expression in vivo by tumor necrosis factor-dependent and -independent pathways. Journal of virology **68:**1265-1270.
88. **Guilhot, S., P. Fowler, G. Portillo, R. F. Margolskee, C. Ferrari, A. Bertoletti, and F. V. Chisari.** 1992. Hepatitis B virus (HBV)-specific cytotoxic T-cell response in humans: production of target cells by stable expression of HBV-encoded proteins in immortalized human B-cell lines. Journal of virology **66:**2670-2678.
89. **Hahn, M., M. J. Nicholson, J. Pyrdol, and K. W. Wucherpfennig.** 2005. Unconventional topology of self peptide-major histocompatibility complex binding by a human autoimmune T cell receptor. Nature immunology **6:**490-496.
90. **Harding, C. V., and E. R. Unanue.** 1990. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. Nature **346:**574-576.
91. **Harty, J. T., A. R. Tvinnereim, and D. W. White.** 2000. CD8+ T cell effector mechanisms in resistance to infection. Annual review of immunology **18:**275-308.
92. **Heemels, M. T., and H. Ploegh.** 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. Annual review of biochemistry **64:**463-491.

93. **Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, and R. A. Ezekowitz.** 1999. Phylogenetic perspectives in innate immunity. Science **284:**1313-1318.
94. **Holler, P. D., L. K. Chlewicki, and D. M. Kranz.** 2003. TCRs with high affinity for foreign pMHC show self-reactivity. Nature immunology **4:**55-62.
95. **Holler, P. D., P. O. Holman, E. V. Shusta, S. O'Herrin, K. D. Wittrup, and D. M. Kranz.** 2000. In vitro evolution of a T cell receptor with high affinity for peptide/MHC. Proceedings of the National Academy of Sciences of the United States of America **97:**5387-5392.
96. **Honda, K., and T. Taniguchi.** 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nature reviews. Immunology **6:**644-658.
97. **Horton, R., L. Wilming, V. Rand, R. C. Lovering, E. A. Bruford, V. K. Khodiyar, M. J. Lush, S. Povey, C. C. Talbot, Jr., M. W. Wright, H. M. Wain, J. Trowsdale, A. Ziegler, and S. Beck.** 2004. Gene map of the extended human MHC. Nature reviews. Genetics **5:**889-899.
98. **Hu, Z., Z. Zhang, E. Doo, O. Coux, A. L. Goldberg, and T. J. Liang.** 1999. Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex. Journal of virology **73:**7231-7240.
99. **Hulsmeyer, M., P. Chames, R. C. Hillig, R. L. Stanfield, G. Held, P. G. Coulie, C. Alings, G. Wille, W. Saenger, B. Uchanska-Ziegler, H. R. Hoogenboom, and A. Ziegler.** 2005. A major histocompatibility complex-peptide-restricted antibody and t cell receptor molecules recognize their target by distinct binding modes: crystal structure of human leukocyte antigen (HLA)-A1-MAGE-A1 in complex with FAB-HYB3. The Journal of biological chemistry **280:**2972-2980.
100. **Hwang, J., L. A. Gheber, L. Margolis, and M. Edidin.** 1998. Domains in cell plasma membranes investigated by near-field scanning optical microscopy. Biophysical journal **74:**2184-2190.
101. **Janeway, C. A., Jr., and R. Medzhitov.** 2002. Innate immune recognition. Annual review of immunology **20:**197-216.
102. **Ji, C., K. S. Sastry, G. Tiefenthaler, J. Cano, T. Tang, Z. Z. Ho, D. Teoh, S. Bohini, A. Chen, S. Sankuratri, P. A. Macary, P. Kennedy, H. Ma, S. Ries, K. Klumpp, E. Kopetzki, and A. Bertoletti.** 2012. Targeted delivery of interferon-alpha to hepatitis B virus-infected cells using T cell receptor-like antibodies. Hepatology.
103. **Jilg, W., C. Delhoune, F. Deinhardt, A. J. Roumeliotou-Karayannis, G. J. Papaevangelou, I. K. Mushahwar, and L. R. Overby.** 1984. Hepatitis B surface antigen (HBsAg) subtype-specific antibodies in persons vaccinated against hepatitis B. Journal of medical virology **13:**171-178.
104. **Jung, M. C., U. Spengler, W. Schraut, R. Hoffmann, R. Zachoval, J. Eisenburg, D. Eichenlaub, G. Riethmuller, G. Paumgartner, H. W. Ziegler-Heitbrock, and et al.** 1991. Hepatitis B virus antigen-specific T-cell activation in patients with acute and chronic hepatitis B. Journal of hepatology **13:**310-317.
105. **Kageyama, S., T. J. Tsomides, Y. Sykulev, and H. N. Eisen.** 1995. Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. J Immunol **154:**567-576.

106. **Kakimi, K., T. E. Lane, S. Wieland, V. C. Asensio, I. L. Campbell, F. V. Chisari, and L. G. Guidotti.** 2001. Blocking chemokine responsive to gamma-2/interferon (IFN)-gamma inducible protein and monokine induced by IFN-gamma activity in vivo reduces the pathogenetic but not the antiviral potential of hepatitis B virus-specific cytotoxic T lymphocytes. The Journal of experimental medicine **194:**1755-1766.
107. **Kalergis, A. M., N. Boucheron, M. A. Doucey, E. Palmieri, E. C. Goyarts, Z. Vegh, I. F. Luescher, and S. G. Nathenson.** 2001. Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. Nature immunology **2:**229-234.
108. **Kieke, M. C., E. V. Shusta, E. T. Boder, L. Teyton, K. D. Wittrup, and D. M. Kranz.** 1999. Selection of functional T cell receptor mutants from a yeast surface-display library. Proceedings of the National Academy of Sciences of the United States of America **96:**5651-5656.
109. **Kieke, M. C., E. Sundberg, E. V. Shusta, R. A. Mariuzza, K. D. Wittrup, and D. M. Kranz.** 2001. High affinity T cell receptors from yeast display libraries block T cell activation by superantigens. Journal of molecular biology **307:**1305-1315.
110. **Konig, R.** 2002. Interactions between MHC molecules and co-receptors of the TCR. Current opinion in immunology **14:**75-83.
111. **Krammer, P. H.** 2000. CD95's deadly mission in the immune system. Nature **407:**789-795.
112. **Krogsgaard, M., and M. M. Davis.** 2005. How T cells 'see' antigen. Nature immunology **6:**239-245.
113. **Krogsgaard, M., K. W. Wucherpfennig, B. Cannella, B. E. Hansen, A. Svejgaard, J. Pyrdol, H. Ditzel, C. Raine, J. Engberg, and L. Fugger.** 2000. Visualization of myelin basic protein (MBP) T cell epitopes in multiple sclerosis lesions using a monoclonal antibody specific for the human histocompatibility leukocyte antigen (HLA)-DR2-MBP 85-99 complex. The Journal of experimental medicine **191:**1395-1412.
114. **Kronenberg, M., G. Siu, L. E. Hood, and N. Shastri.** 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annual review of immunology **4:**529-591.
115. **Laugel, B., J. M. Boulter, N. Lissin, A. Vuidepot, Y. Li, E. Gostick, L. E. Crotty, D. C. Douek, J. Hemelaar, D. A. Price, B. K. Jakobsen, and A. K. Sewell.** 2005. Design of soluble recombinant T cell receptors for antigen targeting and T cell inhibition. The Journal of biological chemistry **280:**1882-1892.
116. **Lebowitz, M. S., S. M. O'Herrin, A. R. Hamad, T. Fahmy, D. Marguet, N. C. Barnes, D. Pardoll, J. G. Bieler, and J. P. Schneck.** 1999. Soluble, high-affinity dimers of T-cell receptors and class II major histocompatibility complexes: biochemical probes for analysis and modulation of immune responses. Cellular immunology **192:**175-184.
117. **Lev, A., G. Denkberg, C. J. Cohen, M. Tzukerman, K. L. Skorecki, P. Chames, H. R. Hoogenboom, and Y. Reiter.** 2002. Isolation and characterization of human recombinant antibodies endowed with the antigen-specific, major histocompatibility complex-restricted specificity of T cells directed

toward the widely expressed tumor T-cell epitopes of the telomerase catalytic subunit. Cancer research **62:**3184-3194.

118. **Li, Y., R. Moysey, P. E. Molloy, A. L. Vuidepot, T. Mahon, E. Baston, S. Dunn, N. Liddy, J. Jacob, B. K. Jakobsen, and J. M. Boulter.** 2005. Directed evolution of human T-cell receptors with picomolar affinities by phage display. Nature biotechnology **23:**349-354.
119. **Li, Z., C. J. Woo, M. D. Iglesias-Ussel, D. Ronai, and M. D. Scharff.** 2004. The generation of antibody diversity through somatic hypermutation and class switch recombination. Genes & development **18:**1-11.
120. **Lin, J., and A. Weiss.** 2001. T cell receptor signalling. Journal of cell science **114:**243-244.
121. **Lord, S. J., R. V. Rajotte, G. S. Korbutt, and R. C. Bleackley.** 2003. Granzyme B: a natural born killer. Immunological reviews **193:**31-38.
122. **Madden, D. R.** 1995. The three-dimensional structure of peptide-MHC complexes. Annual review of immunology **13:**587-622.
123. **Maini, M. K., C. Boni, C. K. Lee, J. R. Larrubia, S. Reignat, G. S. Ogg, A. S. King, J. Herberg, R. Gilson, A. Alisa, R. Williams, D. Vergani, N. V. Naoumov, C. Ferrari, and A. Bertoletti.** 2000. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. The Journal of experimental medicine **191:**1269-1280.
124. **Maini, M. K., C. Boni, G. S. Ogg, A. S. King, S. Reignat, C. K. Lee, J. R. Larrubia, G. J. Webster, A. J. McMichael, C. Ferrari, R. Williams, D. Vergani, and A. Bertoletti.** 1999. Direct ex vivo analysis of hepatitis B virus-specific CD8(+) T cells associated with the control of infection. Gastroenterology **117:**1386-1396.
125. **Mantovani, A., C. Garlanda, A. Doni, and B. Bottazzi.** 2008. Pentraxins in innate immunity: from C-reactive protein to the long pentraxin PTX3. Journal of clinical immunology **28:**1-13.
126. **Mareeva, T., T. Lebedeva, N. Anikeeva, T. Manser, and Y. Sykulev.** 2004. Antibody specific for the peptide.major histocompatibility complex. Is it T cell receptor-like? The Journal of biological chemistry **279:**44243-44249.
127. **Mareeva, T., E. Martinez-Hackert, and Y. Sykulev.** 2008. How a T cell receptor-like antibody recognizes major histocompatibility complex-bound peptide. The Journal of biological chemistry **283:**29053-29059.
128. **Marks, J. D., and A. Bradbury.** 2004. Selection of human antibodies from phage display libraries. Methods Mol Biol **248:**161-176.
129. **Marrack, P., and J. Kappler.** 1987. The T cell receptor. Science **238:**1073-1079.
130. **Marrack, P., K. Rubtsova, J. Scott-Browne, and J. W. Kappler.** 2008. T cell receptor specificity for major histocompatibility complex proteins. Current opinion in immunology **20:**203-207.
131. **Matko, J., Y. Bushkin, T. Wei, and M. Edidin.** 1994. Clustering of class I HLA molecules on the surfaces of activated and transformed human cells. J Immunol **152:**3353-3360.
132. **Matsui, K., J. J. Boniface, P. A. Reay, H. Schild, B. Fazekas de St Groth, and M. M. Davis.** 1991. Low affinity interaction of peptide-MHC complexes with T cell receptors. Science **254:**1788-1791.

133. **Matsuuchi, L., and M. R. Gold.** 2001. New views of BCR structure and organization. Current opinion in immunology **13:**270-277.
134. **Messaoudi, I., J. LeMaoult, and J. Nikolic-Zugic.** 1999. The mode of ligand recognition by two peptide:MHC class I-specific monoclonal antibodies. J Immunol **163:**3286-3294.
135. **Michalak, T. I., P. D. Hodgson, and N. D. Churchill.** 2000. Posttranscriptional inhibition of class I major histocompatibility complex presentation on hepatocytes and lymphoid cells in chronic woodchuck hepatitis virus infection. Journal of virology **74:**4483-4494.
136. **Miller, B. R., S. J. Demarest, A. Lugovskoy, F. Huang, X. Wu, W. B. Snyder, L. J. Croner, N. Wang, A. Amatucci, J. S. Michaelson, and S. M. Glaser.** 2010. Stability engineering of scFvs for the development of bispecific and multivalent antibodies. Protein engineering, design & selection : PEDS **23:**549-557.
137. **Mosmann, T. R., and R. L. Coffman.** 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual review of immunology **7:**145-173.
138. **Murphy, D. B., D. Lo, S. Rath, R. L. Brinster, R. A. Flavell, A. Slanetz, and C. A. Janeway, Jr.** 1989. A novel MHC class II epitope expressed in thymic medulla but not cortex. Nature **338:**765-768.
139. **Murphy, D. B., S. Rath, E. Pizzo, A. Y. Rudensky, A. George, J. K. Larson, and C. A. Janeway, Jr.** 1992. Monoclonal antibody detection of a major self peptide. MHC class II complex. J Immunol **148:**3483-3491.
140. **Muzio, M., D. Bosisio, N. Polentarutti, G. D'Amico, A. Stoppacciaro, R. Mancinelli, C. van't Veer, G. Penton-Rol, L. P. Ruco, P. Allavena, and A. Mantovani.** 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol **164:**5998-6004.
141. **Nayersina, R., P. Fowler, S. Guilhot, G. Missale, A. Cerny, H. J. Schlicht, A. Vitiello, R. Chesnut, J. L. Person, A. G. Redeker, and F. V. Chisari.** 1993. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J Immunol **150:**4659-4671.
142. **Neefjes, J., M. L. Jongsma, P. Paul, and O. Bakke.** 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nature reviews. Immunology **11:**823-836.
143. **O'Herrin, S. M., M. S. Lebowitz, J. G. Bieler, B. K. al-Ramadi, U. Utz, A. L. Bothwell, and J. P. Schneck.** 1997. Analysis of the expression of peptide-major histocompatibility complexes using high affinity soluble divalent T cell receptors. The Journal of experimental medicine **186:**1333-1345.
144. **Oettinger, M. A., D. G. Schatz, C. Gorka, and D. Baltimore.** 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science **248:**1517-1523.
145. **Palmer, E.** 2003. Negative selection--clearing out the bad apples from the T-cell repertoire. Nature reviews. Immunology **3:**383-391.

146. **Paul, W. E., and J. Zhu.** 2010. How are T(H)2-type immune responses initiated and amplified? Nature reviews. Immunology **10:**225-235.
147. **Poisson, F., A. Severac, C. Hourioux, A. Goudeau, and P. Roingeard.** 1997. Both pre-S1 and S domains of hepatitis B virus envelope proteins interact with the core particle. Virology **228:**115-120.
148. **Polakova, K., D. Plaksin, D. H. Chung, I. M. Belyakov, J. A. Berzofsky, and D. H. Margulies.** 2000. Antibodies directed against the MHC-I molecule H-2Dd complexed with an antigenic peptide: similarities to a T cell receptor with the same specificity. J Immunol **165:**5703-5712.
149. **Porgador, A., J. W. Yewdell, Y. Deng, J. R. Bennink, and R. N. Germain.** 1997. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. Immunity **6:**715-726.
150. **Pu, Z., J. A. Carrero, and E. R. Unanue.** 2002. Distinct recognition by two subsets of T cells of an MHC class II-peptide complex. Proceedings of the National Academy of Sciences of the United States of America **99:**8844-8849.
151. **Puri, J., R. Arnon, E. Gurevich, and D. Teitelbaum.** 1997. Modulation of the immune response in multiple sclerosis: production of monoclonal antibodies specific to HLA/myelin basic protein. J Immunol **158:**2471-2476.
152. **Raff, M. C.** 1973. T and B lymphocytes and immune responses. Nature **242:**19-23.
153. **Rajpal, A., N. Beyaz, L. Haber, G. Cappuccilli, H. Yee, R. R. Bhatt, T. Takeuchi, R. A. Lerner, and R. Crea.** 2005. A general method for greatly improving the affinity of antibodies by using combinatorial libraries. Proceedings of the National Academy of Sciences of the United States of America **102:**8466-8471.
154. **Rammensee, H. G., K. Falk, and O. Rotzschke.** 1993. Peptides naturally presented by MHC class I molecules. Annual review of immunology **11:**213-244.
155. **Rang, A., S. Gunther, and H. Will.** 1999. Effect of interferon alpha on hepatitis B virus replication and gene expression in transiently transfected human hepatoma cells. Journal of hepatology **31:**791-799.
156. **Ravetch, J. V., and S. Bolland.** 2001. IgG Fc receptors. Annual review of immunology **19:**275-290.
157. **Ravetch, J. V., and J. P. Kinet.** 1991. Fc receptors. Annual review of immunology **9:**457-492.
158. **Reche, P. A., and E. L. Reinherz.** 2003. Sequence variability analysis of human class I and class II MHC molecules: functional and structural correlates of amino acid polymorphisms. Journal of molecular biology **331:**623-641.
159. **Rehermann, B., P. Fowler, J. Sidney, J. Person, A. Redeker, M. Brown, B. Moss, A. Sette, and F. V. Chisari.** 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. The Journal of experimental medicine **181:**1047-1058.
160. **Rehermann, B., and M. Nascimbeni.** 2005. Immunology of hepatitis B virus and hepatitis C virus infection. Nature reviews. Immunology **5:**215-229.
161. **Reignat, S., G. J. Webster, D. Brown, G. S. Ogg, A. King, S. L. Seneviratne, G. Dusheiko, R. Williams, M. K. Maini, and A. Bertoletti.** 2002. Escaping high

viral load exhaustion: CD8 cells with altered tetramer binding in chronic hepatitis B virus infection. The Journal of experimental medicine **195:**1089-1101.

162. **Reiter, Y., A. Di Carlo, L. Fugger, J. Engberg, and I. Pastan.** 1997. Peptide-specific killing of antigen-presenting cells by a recombinant antibody-toxin fusion protein targeted to major histocompatibility complex/peptide class I complexes with T cell receptor-like specificity. Proceedings of the National Academy of Sciences of the United States of America **94:**4631-4636.
163. **Richman, S. A., D. H. Aggen, M. L. Dossett, D. L. Donermeyer, P. M. Allen, P. D. Greenberg, and D. M. Kranz.** 2009. Structural features of T cell receptor variable regions that enhance domain stability and enable expression as single-chain ValphaVbeta fragments. Molecular immunology **46:**902-916.
164. **Richman, S. A., and D. M. Kranz.** 2007. Display, engineering, and applications of antigen-specific T cell receptors. Biomolecular engineering **24:**361-373.
165. **Richman, S. A., D. M. Kranz, and J. D. Stone.** 2009. Biosensor detection systems: engineering stable, high-affinity bioreceptors by yeast surface display. Methods Mol Biol **504:**323-350.
166. **Robbins, P. F., Y. F. Li, M. El-Gamil, Y. Zhao, J. A. Wargo, Z. Zheng, H. Xu, R. A. Morgan, S. A. Feldman, L. A. Johnson, A. D. Bennett, S. M. Dunn, T. M. Mahon, B. K. Jakobsen, and S. A. Rosenberg.** 2008. Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. J Immunol **180:**6116-6131.
167. **Rodenko, B., M. Toebes, S. R. Hadrup, W. J. van Esch, A. M. Molenaar, T. N. Schumacher, and H. Ovaa.** 2006. Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. Nature protocols **1:**1120-1132.
168. **Rosenberg, S. A.** 2001. Progress in human tumour immunology and immunotherapy. Nature **411:**380-384.
169. **Rudolph, M. G., R. L. Stanfield, and I. A. Wilson.** 2006. How TCRs bind MHCs, peptides, and coreceptors. Annual review of immunology **24:**419-466.
170. **Rudolph, M. G., and I. A. Wilson.** 2002. The specificity of TCR/pMHC interaction. Current opinion in immunology **14:**52-65.
171. **Samuel, C. E.** 2001. Antiviral actions of interferons. Clinical microbiology reviews **14:**778-809, table of contents.
172. **Sastry, K. S., C. T. Too, K. Kaur, A. J. Gehring, L. Low, A. Javiad, T. Pollicino, L. Li, P. T. Kennedy, U. Lopatin, P. A. Macary, and A. Bertoletti.** 2011. Targeting hepatitis B virus-infected cells with a T-cell receptor-like antibody. Journal of virology **85:**1935-1942.
173. **Schatz, D. G., M. A. Oettinger, and D. Baltimore.** 1989. The V(D)J recombination activating gene, RAG-1. Cell **59:**1035-1048.
174. **Schlissel, M. S.** 2003. Regulating antigen-receptor gene assembly. Nature reviews. Immunology **3:**890-899.
175. **Schodin, B. A., C. J. Schlueter, and D. M. Kranz.** 1996. Binding properties and solubility of single-chain T cell receptors expressed in E. coli. Molecular immunology **33:**819-829.
176. **Scholler, J., M. Singh, L. Bergmeier, K. Brunstedt, Y. Wang, T. Whittall, D. Rahman, J. Pido-Lopez, and T. Lehner.** 2010. A recombinant human HLA-

class I antigen linked to dextran elicits innate and adaptive immune responses. Journal of immunological methods **360:**1-9.
177. **Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume.** 2004. Interferon-gamma: an overview of signals, mechanisms and functions. Journal of leukocyte biology **75:**163-189.
178. **Schumacher, T. N.** 2002. T-cell-receptor gene therapy. Nature reviews. Immunology **2:**512-519.
179. **Seeger, C., and W. S. Mason.** 2000. Hepatitis B virus biology. Microbiology and molecular biology reviews : MMBR **64:**51-68.
180. **Sells, M. A., A. Z. Zelent, M. Shvartsman, and G. Acs.** 1988. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. Journal of virology **62:**2836-2844.
181. **Shin, E. C., U. Seifert, T. Kato, C. M. Rice, S. M. Feinstone, P. M. Kloetzel, and B. Rehermann.** 2006. Virus-induced type I IFN stimulates generation of immunoproteasomes at the site of infection. The Journal of clinical investigation **116:**3006-3014.
182. **Shresta, S., C. T. Pham, D. A. Thomas, T. A. Graubert, and T. J. Ley.** 1998. How do cytotoxic lymphocytes kill their targets? Current opinion in immunology **10:**581-587.
183. **Smith, C. A., and T. Kortemme.** 2008. Backrub-like backbone simulation recapitulates natural protein conformational variability and improves mutant side-chain prediction. Journal of molecular biology **380:**742-756.
184. **Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan.** 2009. T cell activation. Annual review of immunology **27:**591-619.
185. **Sobao, Y., K. Sugi, H. Tomiyama, S. Saito, S. Fujiyama, M. Morimoto, S. Hasuike, H. Tsubouchi, K. Tanaka, and M. Takiguch.** 2001. Identification of hepatitis B virus-specific CTL epitopes presented by HLA-A*2402, the most common HLA class I allele in East Asia. Journal of hepatology **34:**922-929.
186. **Sprinzl, M. F., H. Oberwinkler, H. Schaller, and U. Protzer.** 2001. Transfer of hepatitis B virus genome by adenovirus vectors into cultured cells and mice: crossing the species barrier. Journal of virology **75:**5108-5118.
187. **Stavnezer, J., and C. T. Amemiya.** 2004. Evolution of isotype switching. Seminars in immunology **16:**257-275.
188. **Stewart-Jones, G. B., A. J. McMichael, J. I. Bell, D. I. Stuart, and E. Y. Jones.** 2003. A structural basis for immunodominant human T cell receptor recognition. Nature immunology **4:**657-663.
189. **Stone, J. D., M. N. Artyomov, A. S. Chervin, A. K. Chakraborty, H. N. Eisen, and D. M. Kranz.** 2011. Interaction of streptavidin-based peptide-MHC oligomers (tetramers) with cell-surface TCRs. J Immunol **187:**6281-6290.
190. **Subbramanian, R. A., C. Moriya, K. L. Martin, F. W. Peyerl, A. Hasegawa, A. Naoi, H. Chhay, P. Autissier, D. A. Gorgone, M. A. Lifton, K. Kuus-Reichel, J. E. Schmitz, N. L. Letvin, and M. J. Kuroda.** 2004. Engineered T-cell receptor tetramers bind MHC-peptide complexes with high affinity. Nature biotechnology **22:**1429-1434.

191. **Supajatura, V., H. Ushio, A. Nakao, K. Okumura, C. Ra, and H. Ogawa.** 2001. Protective roles of mast cells against enterobacterial infection are mediated by Toll-like receptor 4. J Immunol **167:**2250-2256.
192. **Sykulev, Y., R. J. Cohen, and H. N. Eisen.** 1995. The law of mass action governs antigen-stimulated cytolytic activity of CD8+ cytotoxic T lymphocytes. Proceedings of the National Academy of Sciences of the United States of America **92:**11990-11992.
193. **Takahama, Y.** 2006. Journey through the thymus: stromal guides for T-cell development and selection. Nature reviews. Immunology **6:**127-135.
194. **Takeda, K., T. Kaisho, and S. Akira.** 2003. Toll-like receptors. Annual review of immunology **21:**335-376.
195. **Theofilopoulos, A. N., R. Baccala, B. Beutler, and D. H. Kono.** 2005. Type I interferons (alpha/beta) in immunity and autoimmunity. Annual review of immunology **23:**307-336.
196. **Thomas, S., S. A. Xue, C. R. Bangham, B. K. Jakobsen, E. C. Morris, and H. J. Stauss.** 2011. Human T cells expressing affinity-matured TCR display accelerated responses but fail to recognize low density of MHC-peptide antigen. Blood **118:**319-329.
197. **Thompson, C. B.** 1995. New insights into V(D)J recombination and its role in the evolution of the immune system. Immunity **3:**531-539.
198. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature **317:**489-495.
199. **Toebes, M., M. Coccoris, A. Bins, B. Rodenko, R. Gomez, N. J. Nieuwkoop, W. van de Kasteele, G. F. Rimmelzwaan, J. B. Haanen, H. Ovaa, and T. N. Schumacher.** 2006. Design and use of conditional MHC class I ligands. Nature medicine **12:**246-251.
200. **Tur-Kaspa, R., L. Teicher, O. Laub, A. Itin, D. Dagan, B. R. Bloom, and D. A. Shafritz.** 1990. Alpha interferon suppresses hepatitis B virus enhancer activity and reduces viral gene transcription. Journal of virology **64:**1821-1824.
201. **Udaka, K., K. H. Wiesmuller, S. Kienle, G. Jung, and P. Walden.** 1996. Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. J Immunol **157:**670-678.
202. **Ueda, K., T. Tsurimoto, and K. Matsubara.** 1991. Three envelope proteins of hepatitis B virus: large S, middle S, and major S proteins needed for the formation of Dane particles. Journal of virology **65:**3521-3529.
203. **Unger, W. W., J. Velthuis, J. R. Abreu, S. Laban, E. Quinten, M. G. Kester, S. Reker-Hadrup, A. H. Bakker, G. Duinkerken, A. Mulder, K. L. Franken, R. Hilbrands, B. Keymeulen, M. Peakman, F. Ossendorp, J. W. Drijfhout, T. N. Schumacher, and B. O. Roep.** 2011. Discovery of low-affinity preproinsulin epitopes and detection of autoreactive CD8 T-cells using combinatorial MHC multimers. Journal of autoimmunity **37:**151-159.
204. **Utz, U., D. Banks, S. Jacobson, and W. E. Biddison.** 1996. Analysis of the T-cell receptor repertoire of human T-cell leukemia virus type 1 (HTLV-1) Tax-specific CD8+ cytotoxic T lymphocytes from patients with HTLV-1-associated disease: evidence for oligoclonal expansion. Journal of virology **70:**843-851.

205. **Valenzuela, P., A. Medina, W. J. Rutter, G. Ammerer, and B. D. Hall.** 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature **298:**347-350.
206. **Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia.** 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature **375:**148-151.
207. **van Boxel, G. I., G. Stewart-Jones, S. Holmes, S. Sainsbury, D. Shepherd, G. M. Gillespie, K. Harlos, D. I. Stuart, R. Owens, and E. Y. Jones.** 2009. Some lessons from the systematic production and structural analysis of soluble (alpha)(beta) T-cell receptors. Journal of immunological methods **350:**14-21.
208. **van der Merwe, P. A., and S. J. Davis.** 2003. Molecular interactions mediating T cell antigen recognition. Annual review of immunology **21:**659-684.
209. **Varma, R.** 2008. TCR triggering by the pMHC complex: valency, affinity, and dynamics. Science signaling **1:**pe21.
210. **Wang, H. C., H. C. Wu, C. F. Chen, N. Fausto, H. Y. Lei, and I. J. Su.** 2003. Different types of ground glass hepatocytes in chronic hepatitis B virus infection contain specific pre-S mutants that may induce endoplasmic reticulum stress. The American journal of pathology **163:**2441-2449.
211. **Wang, J., K. Lim, A. Smolyar, M. Teng, J. Liu, A. G. Tse, J. Liu, R. E. Hussey, Y. Chishti, C. T. Thomson, R. M. Sweet, S. G. Nathenson, H. C. Chang, J. C. Sacchettini, and E. L. Reinherz.** 1998. Atomic structure of an alphabeta T cell receptor (TCR) heterodimer in complex with an anti-TCR fab fragment derived from a mitogenic antibody. The EMBO journal **17:**10-26.
212. **Wang, N., D. M. Mattis, E. J. Sundberg, P. M. Schlievert, and D. M. Kranz.** 2010. A single, engineered protein therapeutic agent neutralizes exotoxins from both Staphylococcus aureus and Streptococcus pyogenes. Clinical and vaccine immunology : CVI **17:**1781-1789.
213. **Weaver, C. T., R. D. Hatton, P. R. Mangan, and L. E. Harrington.** 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annual review of immunology **25:**821-852.
214. **Weber, K. S., D. L. Donermeyer, P. M. Allen, and D. M. Kranz.** 2005. Class II-restricted T cell receptor engineered in vitro for higher affinity retains peptide specificity and function. Proceedings of the National Academy of Sciences of the United States of America **102:**19033-19038.
215. **Webster, G. J., S. Reignat, D. Brown, G. S. Ogg, L. Jones, S. L. Seneviratne, R. Williams, G. Dusheiko, and A. Bertoletti.** 2004. Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. Journal of virology **78:**5707-5719.
216. **Wen, J., X. Zhu, B. Liu, L. You, L. Kong, H. I. Lee, K. P. Han, J. L. Wong, P. R. Rhode, and H. C. Wong.** 2008. Targeting activity of a TCR/IL-2 fusion protein against established tumors. Cancer immunology, immunotherapy : CII **57:**1781-1794.
217. **Whitehead, T. A., A. Chevalier, Y. Song, C. Dreyfus, S. J. Fleishman, C. De Mattos, C. A. Myers, H. Kamisetty, P. Blair, I. A. Wilson, and D. Baker.** 2012.

Optimization of affinity, specificity and function of designed influenza inhibitors using deep sequencing. Nature biotechnology **30:**543-548.

218. **Wieland, S., R. Thimme, R. H. Purcell, and F. V. Chisari.** 2004. Genomic analysis of the host response to hepatitis B virus infection. Proceedings of the National Academy of Sciences of the United States of America **101:**6669-6674.
219. **Wieland, S. F., and F. V. Chisari.** 2005. Stealth and cunning: hepatitis B and hepatitis C viruses. Journal of virology **79:**9369-9380.
220. **Willcox, B. E., G. F. Gao, J. R. Wyer, J. E. Ladbury, J. I. Bell, B. K. Jakobsen, and P. A. van der Merwe.** 1999. TCR binding to peptide-MHC stabilizes a flexible recognition interface. Immunity **10:**357-365.
221. **Willcox, B. E., G. F. Gao, J. R. Wyer, C. A. O'Callaghan, J. M. Boulter, E. Y. Jones, P. A. van der Merwe, J. I. Bell, and B. K. Jakobsen.** 1999. Production of soluble alphabeta T-cell receptor heterodimers suitable for biophysical analysis of ligand binding. Protein science : a publication of the Protein Society **8:**2418-2423.
222. **Williams, A. F., and A. N. Barclay.** 1988. The immunoglobulin superfamily--domains for cell surface recognition. Annual review of immunology **6:**381-405.
223. **Wong, P., and E. G. Pamer.** 2003. CD8 T cell responses to infectious pathogens. Annual review of immunology **21:**29-70.
224. **Wu, J. Y., Z. Y. Zhou, A. Judd, C. A. Cartwright, and W. S. Robinson.** 1990. The hepatitis B virus-encoded transcriptional trans-activator hbx appears to be a novel protein serine/threonine kinase. Cell **63:**687-695.
225. **Xue, S. A., L. Gao, D. Hart, R. Gillmore, W. Qasim, A. Thrasher, J. Apperley, B. Engels, W. Uckert, E. Morris, and H. Stauss.** 2005. Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. Blood **106:**3062-3067.
226. **Yewdell, J. W., and J. R. Bennink.** 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annual review of immunology **17:**51-88.
227. **Yewdell, J. W., E. Reits, and J. Neefjes.** 2003. Making sense of mass destruction: quantitating MHC class I antigen presentation. Nature reviews. Immunology **3:**952-961.
228. **Zhao, Y., A. D. Bennett, Z. Zheng, Q. J. Wang, P. F. Robbins, L. Y. Yu, Y. Li, P. E. Molloy, S. M. Dunn, B. K. Jakobsen, S. A. Rosenberg, and R. A. Morgan.** 2007. High-affinity TCRs generated by phage display provide CD4+ T cells with the ability to recognize and kill tumor cell lines. J Immunol **179:**5845-5854.
229. **Zhu, J., H. Yamane, and W. E. Paul.** 2010. Differentiation of effector CD4 T cell populations (*). Annual review of immunology **28:**445-489.
230. **Zhu, X., H. J. Belmont, S. Price-Schiavi, B. Liu, H. I. Lee, M. Fernandez, R. L. Wong, J. Builes, P. R. Rhode, and H. C. Wong.** 2006. Visualization of p53(264-272)/HLA-A*0201 complexes naturally presented on tumor cell surface by a multimeric soluble single-chain T cell receptor. J Immunol **176:**3223-3232.